DYNAMIC REGULATION OF NEUTROPHIL ACTIVATION, MIGRATION AND DEATH

By

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To my grandfather Jin,

there are so many things to catch up on…

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Chapter 1: Introduction

List of Abbreviations

APC	activated protein C
Atg	autophagy-related gene
BK	bradykinin
BSA	bovine serum albumin
CG	cathepsin G
CGD	chronic granulomatous disease
DAMP	damage-associated molecular pattern
DIC	differential interference contrast microscope
DMSO	dimethyl sulfoxide
DPI	diphenyleneiodonium chloride
DVT	deep vein thrombosis
EPCR	endothelial protein C receptor
fMLP	formyl-Met-Leu-Phe
FPR	formyl-peptide receptor
FX(a)	(activated) factor X
FXI(a)	(activated) factor XI
FXII(a)	(activated) factor XII
GAP	GTPase-activating protein
GEF	guanine-nucleotide exchange factor
GPCR	G-protein coupled receptor
H_2O_2	hydrogen peroxide
H3Cit	citrullinated histone H3

HBSS	Hank’s balanced salt solution
HK	high molecular weight kininogen
IL-8	interleukin-8
LC3B	microtubule-associated protein light chain 3B
LPS	lipopolysaccharide
MLC	myosin light chain
MLCK	myosin light chain kinase
mTOR	the mammalian target of rapamycin
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NETosis	neutrophil extracellular traptosis
O_2^-	superoxide anion
PAD4	peptidylarginie deiminase 4
PAK	p21-activated kinase
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PI3K	phosphoinositide 3-kinase
PIP_2	phosphoinosiola-4,5-bisphosphate
PIP_3	phosphoinositol-3,4,5-triphosphate
PK	prekallikrein
PKC	protein kinase C
Plg	plasminogen
PMA	phorbol 12-myristate 13-acetate

PR3	proteinase 3
PRR	pattern-recognition receptor
RA	rheumatoid arthritis
RGD	Arginine-Glycine-Aspartic Acid (Arg-Gly-Asp)
ROCK	Rho-associated kinase
ROS	reactive oxygen species
SEM	standard error of the mean
SLE	systemic lupus erythematous
SOD	superoxide dismutase
TF	tissue factor
TIRF	total internal reflection fluorescence
TNFα	tumor necrosis factor α
ULK	unc-51 like kinase

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Abstract

Dynamic Regulation of Neutrophil Activation, Migration and Death

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Neutrophils, the most abundant white blood cell in the human circulation, play an indispensable role as the first line of innate immune host defense. When circulating neutrophils encounter the signs of infection and/or tissue damage, they rapidly extravasate into the target tissue in order to locally eliminate invading pathogens using their potent microbicidal activity at the cost of their lives. However, improper activation of neutrophils has been shown to mediate the pathological processes of inflammatory diseases and thrombotic complications. This thesis is centered on identifying the key molecular mechanisms that underlie neutrophil activation, migration and death.

Neutrophil migration into/within the inflamed tissue is driven by the cell function termed chemotaxis, in which cell migration is guided by a gradient of chemotatic factors derived from injured tissue or pathogens. For efficient chemotaxis, activated neutrophils develop and maintain the morphological polarization with distinct front and back structures, owing to their highly-coordinated cytoskeletal machinery. While studies have shown that

the Rho-family GTPases, such as Rac, Cdc42 and RhoA GTPases, act as an important cellular compass downstream of chemoattractant receptors, the complex network of signaling pathways downstream of Rho GTPases regulating cytoskeletal dynamics has not been fully characterized. Studies in this thesis suggest that p21-activated kinase (PAK) serves to orchestrate the crosstalk between "frontness" and "backness" signals mediated by Rac/Cdc42 and RhoA, respectively, during neutrophil chemotaxis induced by the bacteria-derived formyl peptide, fMLP.

Upon their arrival to the target tissue, neutrophil activation triggers their professional microbicidal program, including an active form of cell death by the release of neutrophil extracellular traps (NETs). NETs consist of decondensed nuclear chromatin decorated with antibacterial proteins that together form a physical trap for pathogen killing; however, physiological stimuli and cellular events required for NETs formation are ill-defined. We show that the mammalian target of rapamycin (mTOR) pathway, downstream of fMLP signaling, plays a central role in the regulation of neutrophil fate towards NETs formation.

The activation of innate immunity and blood coagulation is linked as effectors of host defense response, where local accumulation of neutrophils is shown to promote prothrombotic conditions. In this thesis, studies reveal that the activated coagulation factor XI (FXIa) displays an inhibitory effect on neutrophil activation and chemotaxis triggered by chemotactic stimuli including fMLP. This novel interaction between

neutrophils and FXIa implicates a potential role of FXIa in modulating innate immunity at the interphase of inflammation and thrombosis.

Collectively, this dissertation provides novel insights into the tight regulation of neutrophil activation, migration and fate decision, which constitute a crucial mechanism in health and disease.

Chapter 1: Introduction

1.1 Innate Immunity

Innate immunity is a conserved host defense mechanism displayed by all multicellular organisms and mediated via germline-coded receptors that recognize components derived from foreign pathogens and/or tissue damage (Medzhitov and Janeway 1997). In contrast, adaptive immunity, which is found only in vertebrates, is characterized by the development of antigen-specific receptors via gene rearrangement in lymphocytes in response to signals provided by innate immune cells. Evolutionarily, innate immunity has been selected for based on the ability of immune cells to immediately recognize the molecular structures shared by a large group of pathogens, namely pathogen-associated molecular patterns (PAMPs: e.g. microbial peptides, nucleic acids and lipoproteins), or damage-associated molecular patterns (DAMPs: e.g. nucleotides and heat shock proteins) released from injured cells. PAMPs and DAMPs are detected by pattern-recognition receptors (PPRs) expressed on innate immune cells, such as tissue-resident macrophages and dendritic cells. This process triggers intracellular signaling cascades leading to subsequent inflammatory responses. Local inflammation is further amplified by the recruitment of circulating leukocytes, such as neutrophils and monocytes, which employ multiple tactics for pathogen clearance in response to PAMPs/DAMPs (Medzhitov and Janeway 1997; Newton and Dixit 2012). At sites of inflammation, activated innate immune cells play a critical role in directly eliminating pathogens through phagocytosis or secretion of oxidants and microbicidal peptides, while providing co-stimulatory

molecules and effector cytokines required for antigen recognition by adaptive immune cells.

Among the leukocyte subpopulations, it is well established that neutrophils play an important role in the early stage of innate immunity. For instance, depletion of neutrophils in mice at early time points (1~3 days) following the injection of one third the 50% lethal dose of *Listeria moocytogenes* resulted in a profound decrease in survival (Rogers and Unanue 1993). Moreover, patients with low neutrophil counts, or neutrophil dysfunction due to chronic granulomatous disease (CGD) or leukocyte adhesion deficiency, display severe susceptibility to bacterial and fungal infection (Borregaard 2010; Summers *et al.* 2010). Neutrophils are terminally differentiated in the bone marrow at a production rate of 5-10 $\times$ 10^{10} cells/day in normal human adults, and are released from the hematopoietic compartment to the vascular compartment. In the absence of infection or injury, neutrophils patrol both the circulation and temporally reside in the marginated pool (liver, bone marrow or spleen) in search of PAMPs/DAMPs. Neutrophils have a half-life of 6-8 h in the circulation, and are removed by macrophages of the reticuloendotheial system. When microorganisms successfully invade tissues, circulating neutrophils rapidly migrate across the endothelial linings and into the inflamed tissue, where they elicit microbicidal activity and undergo cell death, either by apoptosis or neutrophil extracellular traposis (NETosis), as illustrated in Figure 1.1.

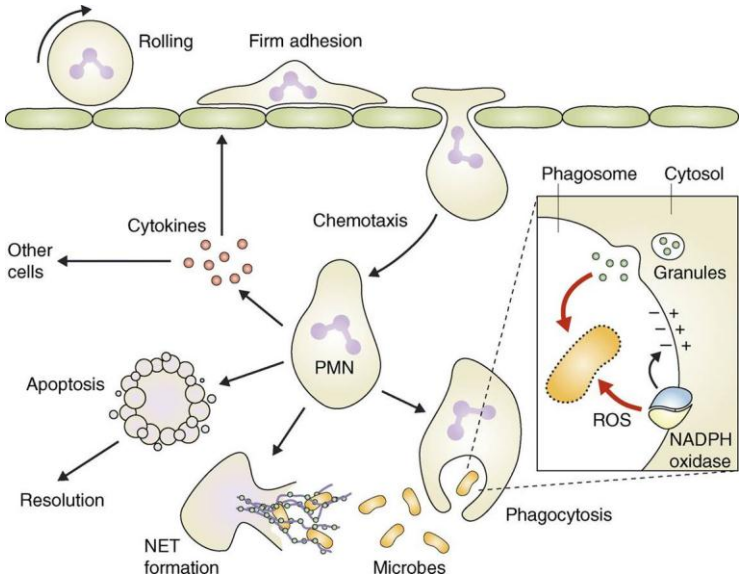

Figure 1.1 Neutrophil functions.

After migrating to the site of inflammation, neutrophils (PMN) phagocytose and digest invading microbes; release NETs, which likely trap bacteria; and produce cytokines, which contribute to the inflammatory reaction. Once the infection is cleared, neutrophils die by apoptosis and trigger an active program to resolve inflammation. Inset, pathogen killing inside the phagosome occurs by ROS generated by the NADPH oxidase, as well as by granule enzymes released from intracellular granules. Figure was adapted from ©Mocsai et al., 2013, originally published in J Exp Med 210(7): 1283-99.

The traditional view of neutrophil function maintains that these cells are no more than simply “suicide killers”, owing to their major roles in locally eliminating pathogens at the cost of their lives. However, recent studies have uncovered a set of complex and highly regulated functions for neutrophils in immunity and beyond. For instance, neutrophils have been found to participate in the regulation of allergic reactions, as well as adaptive immune components (Nathan 2002; Mocsai 2013). Moreover, their ability to release granular enzymes and neutrophil extracellular traps (NETs) represents a unique strategy for host defense (Borregaard 2010). Despite the protective roles of neutrophils against infectious microbes, dysregulation of neutrophil function may also lead to exaggerated inflammation and collateral tissue damage, and thus consequently contribute to the

pathology of autoimmune diseases, anaphylaxis and other clinical complications (Mocsai 2013). Therefore, the tight regulation of neutrophil activation, tissue recruitment and fate decision constitute a crucial mechanism in health and disease.

1.2 Neutrophil Tissue Recruitment and Microbicidal Functions

Neutrophil transmigration often takes place within postcapillary venules, where the physical parameters of the vasculature promote the interaction of neutrophils with endothelial cells (Witko-Sarsat *et al.* 2000; Borregaard 2010). Inflammation induces the expression of various adhesion molecules and chemokines on the endothelial cell surface. Initial capture and rolling of neutrophils is mediated by selectins and integrins, allowing neutrophil activation by chemokines presented on the endothelium. Neutrophil firm adhesion is established through the binding of activated leukocyte β2 integrin, LFA-1 (αLβ2), to endothelial adhesion molecule ICAM-1 (Phillipson *et al.* 2006; Sarantos *et al.* 2008). Adhesion-mediated guidance drives intravascular crawling, in a β2 integrin Mac-1 (αMβ2)-dependent manner (Phillipson *et al.* 2006), through the barriers of endothelial cells, endothelial basement membrane and the pericyte sheath.

Upon their extravasation into the tissue, neutrophils employ multiple tactics for pathogen killing (Kolaczkowska and Kubes 2013; Mocsai 2013). This section summarizes the state-of-the-art understanding of the molecular mechanisms underlying two of the key neutrophil functions: chemotaxis and NETs formation.

1.2.1 Chemotaxis

Chemotaxis is the driving force of efficient transmigration and migration within tissues, guided by a successive combination of chemoattractants. The gradients of chemoattractants are formed by factors released from inflamed endothelial cells and stromal cells (e.g. interleukin-8; IL-8, leukotriene B4, platelet-activating factor), and the "end-target" factors derived from pathogens and dying cells (e.g. formyl-Met-Leu-Phe peptides; fMLP, complement C5a) in local tissues (Witko-Sarsat *et al.* 2000; Phillipson and Kubes 2011). These chemoattractants trigger intracellular signaling cascades via specific G protein-coupled receptors (GPCRs), whereby a hierarchy of chemotactic signals directs neutrophils toward the bacterial or necrotic focus (Heit *et al.* 2002; McDonald *et al.* 2010; Kim and Haynes 2012). Upon exposure to chemoattractants, neutrophils undergo a dramatic morphological polarization and develop a distinct front structure, termed the lamellipodia, and a rear structure, termed the uropod. The efficient migratory ability of neutrophils (up to 30 μm/min) is established by the coordinated crosstalk between "frontness" and "backness" signals, mediated by actin-dependent membrane protrusion and myosin-dependent contraction, respectively.

Formyl-peptide receptors: The family of formyl peptide receptors (FPRs) plays an essential role in inflammatory events associated with antibacterial host defense, as well as non-bacterial, DAMPs-induced sterile tissue injury. Mice lacking FPRs display rapid and increased mortality after *Listeria* infection (Gao *et al.* 1999). Genetic deletion of FPRs in mice, as well as blockade of neutrophil FPRs by inhibitory antibodies or selective antagonists *in vitro*, resulted in random unidirectional migration within the necrotic zone

(McDonald *et al.* 2010). FPR binds to fMLP derived from bacteria or the mitochondria of dying cells, or host-derived agonists such as antimicrobial cathelicidin LL-37, amyloidgenic proteins, or neutrophil protease cathepsin G (Rabiet *et al.* 2007), leading to the activation of heterotrimeric G protein and a complex set of downstream signaling pathways.

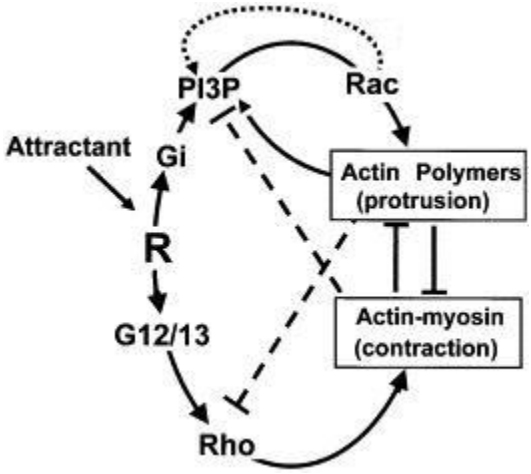

Figure 1.2 A model of neutrophil cytoskeletal dynamics

After the binding of attractant to surface GPCR (R), Gα subunit (G_i and $G_{12/13}$) of heterotrimeric G proteins activates downstream effectors. PI3P is generated by PI3K and activates Rac-mediated actin protrusion at the leading edge (frontness signal), while Rho GTPases promote actomyosin contraction at the uropod (backness signal). Polarity is maintained by the antagonizing signals between frontness and backness signals. Figure was adapted from © 2003 Cell Press, originally published in *Cell* (Xu *et al*, 2003).

Rho family of small GTPases: Rho GTPases, including Rac, Cdc42 and RhoA, have been shown to play an important role in neutrophil cytoskeletal dynamics. Rho GTPases act as molecular switches that cycle between a GTP-bound active form and GDP-bound inactive form via guanine nucleotide exchange factors (GEFs) and GTPase-activating factors (GAPs) (Bokoch 2005). GPCR activation leads to the dissociation of heterotrimeric G protein into Gα and Gβγ subunits that activate downstream effectors

such as GEFs for Rac (P-Rex1, DOCK2 and Vavs), Cdc42 (PIXα) and RhoA (PDZ-GEF and Lsc) (Li *et al.* 2003; Gakidis *et al.* 2004; Dong *et al.* 2005; Francis *et al.* 2006; Kunisaki *et al.* 2006; Wong *et al.* 2007; Lawson *et al.* 2011). Activated Rac and Cdc42 GTPases initiate peripheral actin polymerization by activating the proteins of the WASP/WAVE family and promoting Arp2/3 complex-driven branching of actin filaments (Cory and Ridley 2002; Sun *et al.* 2007; Kumar *et al.* 2012), driving the "frontness" signal in migrating neutrophils. Studies using Rac1 and/or Rac2 knockout mice have highlighted the role of Rac1 in polarization and directionality, as well as the role of Rac2 in stable F-actin assembly and motility (Roberts *et al.* 1999; Glogauer *et al.* 2003; Gu *et al.* 2003; Sun *et al.* 2004; Zhang *et al.* 2009). In contrast, inhibition of RhoA, its effector Rho-associated kinase (ROCK) or myosin II has been shown to result in the formation of multiple lamellipodia and long uropods in neutrophils (Alblas *et al.* 2001; Xu *et al.* 2003; Wong *et al.* 2007). As ROCK phosphorylates myosin light chain at Ser 19 to increase myosin II ATPase activity for actomyosin contractility, these observations suggest that the RhoA-ROCK axis regulates myosin II-dependent uropod retraction, or the neutrophil "backness" signal. In addition, it has been shown that antagonistic signals between Rac/Cdc42 and RhoA serve to stabilize the polarity of migrating cells (Bokoch 2005) (Figure 1.2).

Phosphoinositide 3-kinase: After GPCR stimulation, G protein βγ subunits activate phosphoinositide 3-kinase (PI3K), which converts the membrane lipid phosphoinositol-4,5-bisphosphate (PIP_2) into phosphoinositol-3,4,5-triphosphate (PIP_3) within 10 seconds after fMLP-FPR ligation (Stephens *et al.* 1991). In contrast to the unaltered subcellular

localization of chemoattractant GPCR receptors upon activation (Servant *et al.* 1999; Jin *et al.* 2000), PIP_3 accumulation to the leading edge has been recognized as an early event during chemotaxis of primary neutrophils, neutrophil-like cell lines and *Dictyostelium* cells (Parent *et al.* 1998; Meili *et al.* 1999; Servant *et al.* 2000). Experiments utilizing both genetic manipulation and pharmacological inhibition of *Dictyostelium* and neutrophils have revealed that PI3K activity and PIP_3 gradients play key roles in polarization and cell motility, although these pathways are dispensable for gradient sensing and orientation (Andrew and Insall 2007; Ferguson *et al.* 2007; Hoeller and Kay 2007; Nishio *et al.* 2007). PI3K/PIP_3 effectors in neutrophils include the GEFs, such as P-Rex1, Vavs and PIXα, which mediate the frontness polarity via Rac or Cdc42 activation (Stephens *et al.* 2008). Leading edge dynamics are stabilized via the positive feedback loop of Rac/Cdc42 and PI3K (Servant *et al.* 2000; Weiner *et al.* 2002; Srinivasan *et al.* 2003).

Traditional views of Rho GTPases or PI3K as a single "cellular compass" have been challenged by recent discoveries; for instance, recent evidence has implicated that neutrophil chemotaxis requires a complex network of coupled signaling crosstalk and feedback loops. Further investigations are required to fully elucidate the key mechanisms of chemotaxis, such as the interactions among GEFs/GAPs, downstream effectors of Rho GTPases, and cytoskeletal modules, under physiological contexts.

1.2.3 Neutrophil extracellular traps

Researchers in the field of neutrophil biology have long observed unusual extracellular fiber-like structures derived from neutrophils; however, these observations were largely regarded as experimental artifacts. It was not until 2004 that Brinkmann *et al.* reported that neutrophils, upon their activation, can release intracellular granule proteins and chromatin that together form neutrophil extracellular traps (NETs) to kill bacteria (Brinkmann *et al.* 2004). Since then, NETs have been highlighted as a new paradigm in innate immunity. NETs consist of decondensed chromatin fibers decorated with antimicrobial proteins (histones, cathelicidins, defensins) and proteases (neutrophil elastase; NE, proteinase 3; PR3, cathepsin G; CG) (Knight *et al.* 2012). This extracellular web-like structure functions as a physical trap for pathogens in tissues to directly kill trapped pathogens by antimicrobial factors and/or present them to other phagocytic cells. As a form of active cell death, NETs allow neutrophils to exert a bactericidal function beyond their lifetime, distinct from the apoptotic cell death program.

NETs in diseases: Besides their antimicrobicidal activity, recent research has identified links between NETs and various clinical complications, as summarized in Table 1.1. Along these lines, extracellular chromatin or histones in the circulation has been found to be associated with the severity of sepsis (Xu *et al.* 2009), sterile inflammatory liver injury (Huang *et al.* 2011) and stroke (De Meyer *et al.* 2012). It has been hypothesized that under these conditions, NETs may serve as a source of endogenous chromatin. The pathological mechanisms by which NETs contribute to clinical complications are proposed as follows: (1) NETs-associated proteins exposed to the extracellular

environment display cytotoxic activity on surrounding cells such as neurons, endothelial and epithelial cells (Gupta *et al.* 2010; Allen *et al.* 2012; Saffarzadeh *et al.* 2012); (2) NETs present autoantigens that can cause autoimmunity and chronic inflammation (Knight *et al.* 2012); (3) NETs provide a platform for the activation of coagulation factors and platelets (Ma and Kubes 2008; Kambas *et al.* 2012; von Bruhl *et al.* 2012); and (4) NETs capture circulating blood and tumor cells (Fuchs *et al.* 2010; Cools-Lartigue *et al.* 2013).

Table 1.1 NETs-associated conditions

Condition	Species (organ)	Reference
Sepsis	Mouse (liver, lung) Human (patient PMN in vitro)	McDonald *et al*, 2012 Kambas *et al*, 2012
Pneumonia	Mouse (airway PMN in vitro)	Douda *et al*, 2011
Necrotizing fasciitis	Mouse (skin)	LI *et al*, 2010
Small vessel vasculitis	Human (kidney)	Kassenbrock *et al*, 2009
Systemic lupus erythematosus	Human (patient PMN in vitro)	Hakkim *et al,* 2010; Leffler *et al,* 2012
Psoriasis	Human (skin)	Lin *et al*, 2011; Skrzeczynska-Moncznik *et al*, 2012
Gout	Human (synovial fluid)	Mitroulis *et al*, 2011
Deep vein thrombosis	Mouse (thrombi) Baboon (thrombi) Human (thrombi)	Von Bruhl *et al*, 2012; Brill *et al*, 2012; Matrinod *et al,* 2010 Fuchs *et al*, 2010 Nakazawa *et al*, 2012
Atherosclerosis	Mouse (artery)	Doering *et al*, 2012
Acute brain injury	Mouse (cerebral cortex)	Allen *et al*, 2012
Cancer metastasis (with systemic infection)	Mouse (liver, lung) Human (in vitro)	Cools-Lartigue *et al*, 2013

Mechanism of NETs formation: The process of NETs release, so-called NETosis, is characterized by chromatin decondensation, in contrast to chromatin condensation during apoptosis. Studies have identified several cellular events required for NETosis prior to chromatin decondensation. For example, in response to various inflammatory stimuli, neutrophils rapidly produce reactive oxygen species (ROS) via NADPH oxidase (Borregaard 2010). Neutrophils obtained from CGD patients, who lack functional NADPH oxidase, displayed the inability to form NETs *in vitro* (Remijsen *et al.* 2011), suggesting a crucial role of ROS production during NETosis. In the same study, the authors also reported that autophagic activity shapes the other essential axis of early NETosis, as the inhibition of NADPH oxidase or autophagy led to chromatin condensation and apoptotic death (Remijsen *et al.* 2011). In the later stage of NETosis, the Ca^{2+}-dependent peptidylarginine deiminase 4 (PAD4) has been shown to play an essential role in chromatin decondensation via its activity for the posttranslational modification of histones, namely citrullination (Neeli *et al.* 2008; Wang *et al.* 2009; Li *et al.* 2010). PAD4-mediated histone citrullination alters the electrostatic charge on arginine residues of histone tails, resulting in loosening of the chromatin structures. In addition, the neutrophil granule enzymes, NE and myeloperoxidase, have also been shown to digest nucleosomal histones and promote chromatin decondensation (Papayannopoulos *et al.* 2010). However, the signaling pathways that link these cellular events have not yet been explained. Intracellular Ca^{2+} release from the endoplasmic reticulum and NADPH-driven ROS production has been hypothesized to cooperatively contribute to PAD4 activation and histone citrullination (Rohrbach *et al.* 2012). Under *in vitro* settings, the Raf-MEK-ERK pathway and protein kinase C (PKC) have been shown to be involved in

NETosis upstream of NADPH oxidase activation (Hakkim *et al.* 2010; Neeli and Radic 2013), while murine deficiency in Rac2 GTPase, but not Rac1 deficiency, resulted in impaired NETs formation due to the lack of ROS production (Lim *et al.* 2011).

After the clearance of infectious agents, neutrophil death switches from NETosis to apoptosis in order to avoid excessive inflammatory states. Therefore, understanding the molecular mechanisms underlying neutrophil fate decisions are key to elucidating the role of NETs in health and disease.

1.3 Bidirectional Relationship between Inflammation and Thrombosis

Mammalian hemostasis involves two principle components; the coagulation system and platelets. Both components act in concert to generate a hemostatic clot that forms a seal at the site of vascular injury. In addition, as shown in studies using a murine model of bacterial infection (Luo *et al.* 2011; Luo *et al.* 2013), several molecules in the coagulation system are required to restrict pathogen dissemination and promote pathogen clearance by innate and adaptive immunity. Immune cell activity has been shown to regulate pathological clot formation (i.e. thrombosis), termed "immunothrombosis", whereby the local formation of clots in microvessels constitutes an intrinsic effector mechanism of innate immunity (Engelmann and Massberg 2013). The coupled activation of inflammation and thrombosis is hypothesized to play an important role in the pathogenesis of vascular diseases (Levi and van der Poll 2005). Although multiple immune components are known to mediate this crosstalk, the following section focuses primarily on the roles of neutrophils in thrombosis and hemostasis.

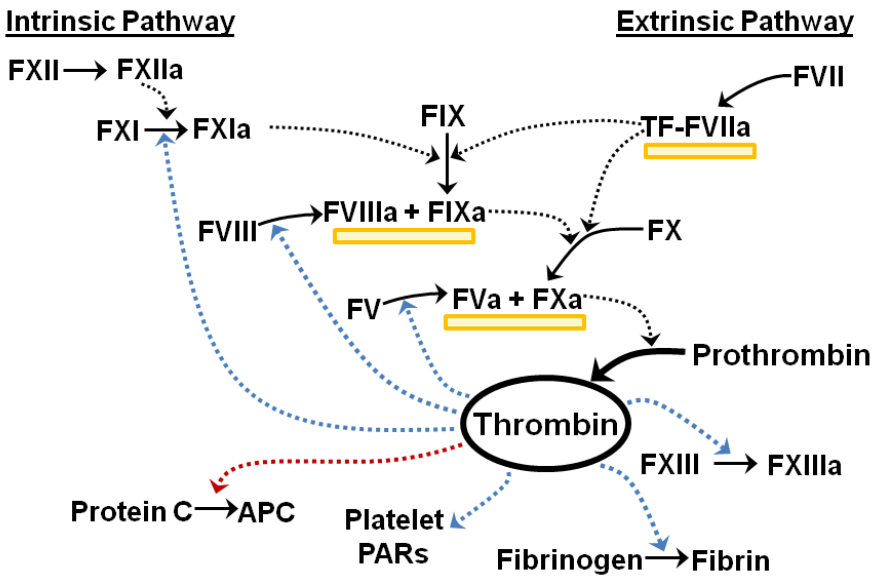

Figure 1.3 Coagulation pathways.

Coagulation is triggered by upstream activation of the extrinsic pathway via tissue factor (TF), or the intrinsic pathway via FXIIa, resulting in thrombin generation through FXa cleavage of prothrombin on the cell membrane surface (yellow boxes). The enzymatic functions of thrombin and other coagulation enzymes are indicated by dashed lines. Procoagulant actions of thrombin are indicated by blue lines and anticoagulant actions by red lines.

Hemostasis: Figure 1.3 depicts the process of hemostatic clot formation. Upon vascular injury, platelets in the circulation are recruited to the site of injury through their specific surface receptors for endothelial ligands (Versteeg *et al.* 2013). In parallel, the exposure of tissue factor (TF) expressed on subendothelial tissues to blood initiates the extrinsic coagulation pathway, sequentially generating activated factor X (FXa) and thrombin, which subsequently cleaves fibrinogen to form fibrin. Efficient activation of coagulation proteases requires the platelet surface to assemble coagulation factor complexes. In the intrinsic coagulation pathway, activated factor XII (FXIIa) leads to a subsequent activation of factor XI (FXI), factor IX (FIX), leading to FXa and thrombin generation. Alternatively, thrombin generated via the extrinsic pathway can activate FXI to form an amplification loop of thrombin generation leading to clot formation.

Inflammation-induced prothrombotic activity: Inflammatory cytokines such as interleukin-6 can trigger the expression of TF on monocytes (Levi and van der Poll 2005), while neutrophils express TF in response to stimuli such as fMLP, compliment C5a (Ritis *et al.* 2006), following P-selectin ligation (Maugeri *et al.* 2006), or upon vascular injury (Darbousset *et al.* 2012). *In vivo* research has shown that the association of TF-expressing leukocytes with thrombosis during sepsis, and following atherosclerotic plaque rupture (Libby and Aikawa 2002). In addition, as discussed in section 2.2, NETs have been shown to play an important role in the development of thrombotic complications by activating both intrinsic (von Bruhl *et al.* 2012) and extrinsic (Kambas *et al.* 2012) pathways, as well as activating platelets (Fuchs *et al.* 2010). In support of the findings from animal thrombosis models, Nakazawa *et al.* have identified NETs in thrombi derived from a patient diagnosed with microscopic polyangiitis and complicated with deep vein thrombosis (DVT) (Nakazawa *et al.* 2012). Moreover, the neutrophil granule enzyme CG has been shown to directly generate FXa (Plescia, Biochem 1996), while NE can cleave and deactivate tissue factor pathway inhibitor, which inhibits FXa activity, thus promoting a prothrombotic phenotype (Massberg *et al.* 2010).

Thrombosis-induced inflammatory activity: Circulating leukocytes, including neutrophils, are recruited to thrombi via P-selectin expressed on clot-bound platelets, whereas the systemic detection of heterotypic leukocyte-platelet aggregates has been suggested as a marker of ongoing vascular thrombosis and inflammation (Furman *et al.* 2001; Sarma *et al.* 2002). Within thrombi, platelets generate a gradient of the chemokine NAP-2 to drive intravascular migration of neutrophils (Ghasemzadeh *et al.* 2013). Products of the

activated coagulation pathways, for instance thrombin and FXa, can induce leukocyte migration as well as a wide range of proinflammatory responses in leukocytes, via expression of adhesion molecules and cytokines (Delvaeye and Conway 2009). Conversely, the anticoagulant protein C pathway, in which protein C is cleaved by thrombin to form activated protein C (APC; Fig 1.3), plays a key role in anti-inflammatory activity in addition to its role as an endogenous anticoagulant via inactivation of factor Va and VIIIa. Studies have identified the ability of APC to inhibit neutrophil chemotaxis driven by fMLP, IL-8 or C5a, via neutrophil-expressed endothelial protein C receptor (EPCR) (Sturn *et al.* 2003) and/or β1/β3 integrins (Elphick *et al.* 2009). Furthermore, the administration of recombinant APC significantly reduced leukocyte infiltration to lungs in an endotoxin-induced human model of acute pulmonary inflammation (Nick *et al.* 2004). Of note, EPCR expression is downregulated during inflammation due to neutrophil enzyme PR3-mediated proteolytic degradation (Villegas-Mendez *et al.* 2007), implying a vicious circle in the context of a thrombotic/inflammatory microenvironment.

Neutrophils as a therapeutic target: Given that neutrophil functions can contribute to thrombosis as discussed above, specific targeting of neutrophil-driven inflammation may represent a therapeutic strategy to combat thrombosis associated with inflammation. In the context of neutrophilic inflammation such as chronic obstructive pulmonary disease and acute lung injury, several drugs that target neutrophil function are currently under clinical trials (Barnes 2007). These include the antagonists for chemoattractant receptors (e.g. IL-8 receptor antagonist; GSK-656933), chemoattractant generation (e.g.

leukotriene B4-generating 5'-lipoxygenase antagonist; Zileuton) or adhesion molecules (e.g. selectin inhibitor; Bimosiamose). Additional therapeutic strategies include targeting key signaling pathways such as MAPKs (Arthur and Ley 2013) and NF-κB (Barnes 2007). However, the therapeutic window for these agents should be carefully investigated in a context-dependent manner, in order to avoid increased susceptibility to infection and off-target effects other than neutrophil responses. Along these lines, pre-clinical studies using pharmacological inhibitors and knockout mice have highlighted PAD4, expressed primarily in neutrophils, as a promising therapeutic target for inflammatory and/or thrombotic conditions by suppressing NETs formation (Rohrbach *et al.* 2012). Moreover, neutrophil interactions with endothelial cells and the fibrinolytic pathways also mediate the interplay between inflammation and thrombosis (Levi and van der Poll 2005; Engelmann and Massberg 2013), which were not discussed in this section. Further understanding of the crosstalk between inflammation and thrombosis as an integrated system will provide insights into therapeutic intervention to combat these pathological conditions.

1.4 Thesis Overview

Neutrophils serve as a "double-edged sword" in innate immunity, with their ability to protect the host from invading pathogens and their ability to induce pathologic tissue damage. This thesis focuses on elucidating the regulatory mechanisms underlying the major neutrophil functions of chemotaxis and NETs formation, and on the characterization of a novel neutrophil regulator that mediates the interplay between inflammation and coagulation.

Although previous studies have delineated the molecular players that underlie the steps of neutrophils chemotaxis, such as directional sensing, polarization and motility, the manner in which cytoskeletal dynamics are spatially and temporally coordinated to drive efficient chemotaxis remain ill-defined. Studies in Chapter 3 highlight a potential role of the Rac/Cdc42 effector, p21-activated kinase (PAK), in the crosstalk between Rho GTPases during fMLP-driven cytoskeletal reorganization.

After migration along chemoattractant gradients, the release of NETs immobilizes pathogens and facilitates their destruction. However, the exposure of NETs-associated antimicrobial proteins can be detrimental for host tissue. Despite recent reports demonstrating NETs-related disease pathology, the triggers for NETs formation are still under intensive debate, and it seems that multiple signaling pathways and cellular events are required to lead neutrophils towards this specific form of programmed death. Studies in Chapter 4 describe the pivotal role of the mammalian target of rapamycin (mTOR) pathway in the neutrophil fate decision of NETosis in response to fMLP.

Host defense responses utilize the ability of the coagulation system to restrict pathogens to local sites, where inflammatory cells encounter blood clots packed with coagulation factors and platelets. Building on previous reports of coagulation-mediated modulation of inflammation, studies in Chapter 5 characterize the interaction between activated coagulation factor XI (FXIa) and neutrophils, and highlight a potential role for FXIa in the control of leukocyte tissue trafficking.

The studies outlined in Chapters 3-5 provide new insights into the regulatory mechanisms of neutrophil function. In Chapter 6, the key findings from my thesis research are summarized and areas of interest for future work are highlighted.

Chapter 2: Common Materials and Methods

2.1 Ethical Considerations

Studies in this thesis were conducted using human blood. All human donors were healthy and gave full informed consent in accordance with the Declaration of Helsinki. Experiments using human donors were performed with approval of the Oregon Health & Science University Institutional Review Board.

2.2 Common Reagents

Polymorphprep was from Axis-Shield PoC AS (Olso, Norway). Hank's Balanced Salt Solution (HBSS) was from Corning (Manassas, VA, USA). Fluo-4 AM was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.3 Blood Collection and Neutrophil Isolation

2.3.1 Blood collection

Human venous blood was collected by venipuncture from healthy adult male and female volunteers directly into syringes containing the anticoagulant, citratephosphate-dextrose (1:7 vol/vol).

2.3.2 Neutrophil purification

Blood was layered over an equal volume of Polymorphprep and centrifuged at 500 g for 45 min at 18°C. The lower layer containing neutrophils was collected and washed with

HBSS by centrifugation at 400 g for 10 min. To remove contaminating red blood cells, the pellet was resuspended in sterile H_2O for 30 s, followed by the immediate addition of 10× PIPES buffer (250 mM PIPES, 1.1 mM NaCl, 50 mM KCl, pH 7.4). After centrifugation at 400 g for 10 min, the pellet was resuspended in PMN buffer [HBSS containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% w/v bovine serum albumin (BSA)].

2.4 Live-cell Imaging

2.4.1 Insall chemotaxis chamber assays

Direct visualization of chemotaxis generates an informative data set that can be quantified to define the different aspects of cell migration such as speed, turning rate, orientation, and morphology/polarity (Sumen *et al.* 2004). Several experimental chambers for live-cell chemotaxis imaging have been developed (Figure 2.1). The original chemotaxis chamber, named the Zigmond chamber, presents a limitation in that it can only establish a short-lived, unstable linear gradient. In contrast, the Dunn chamber creates a stable but non-linear gradient, however, is not compatible with high-resolution imaging. Recently, the Insall chamber has been developed for use in high-resolution live-cell imaging with a long-lived linear gradient (Muinonen-Martin *et al.* 2010; Muinonen-Martin *et al.* 2013).

For the visualization of neutrophil chemotaxis using the Insall chamber, acid-washed, 22 mm square coverslips (#1.5, 0.16–0.18 mm) were coated with fibronectin (20 μg/ml) for 1 h and blocked with heat-inactivated BSA (0.05% w/v) for 1 h. Purified neutrophils were incubated at 37°C for 30 min on the coverslips. Inner and outer wells of an Insall

chamber were filled with vehicle (PMN buffer), and a coverslip with adherent cells was inverted onto the chamber. After the vehicle solution in outer wells was aspirated, fMLP-containing medium was added. Images were obtained every 10 s for 30 min at 40× using differential interference contrast (DIC) microscopy. In selected experiments, cells were pretreated with indicated reagents at room temperature prior to the experiment. Images were analyzed using ImageJ plugin MTrackJ and quantified using the CircStat toolbox (Berens 2009) for MATLAB (MathWorks, Natick, MA, USA).

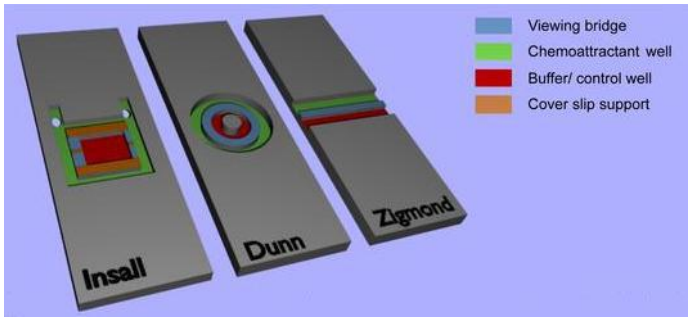

Figure 2.1 Comparison of bridge chamber features.

(A) Schematic showing the Insall, Dunn and Zigmond chambers. The wells for chemoattractant (green) and buffer/control (red) have been color-coded for direct comparison, along with the viewing bridges (blue) and cover slip supports (orange). Figure was adapted from ©Muinonen-Martin et al., 2013, originally published in PLOS ONE 5(12): e15309.

2.4.2 Intracellular Ca^{2+} measurement

Human neutrophils (2×10^6 /ml) were loaded with the intracellular Ca^{2+} dye, fluo-4 AM (final 2 μM) and plated on a fibronectin-coated surface for 30 min at 37°C. After washing with Ca^{2+}-free HBSS, cells were incubated for another 30 min at 37°C in PMN buffer and treated with indicated agents. Intracellular Ca^{2+} spikes in neutrophils were monitored for 15 min at 40× using a Zeiss Axiovert fluorescent microscopy, and analyzed using a custom MATLAB program.

2.5 Immunofluorescence Microscopy

Purified human neutrophils (2×10^6 /ml) were incubated on fibronectin-coated surfaces at 37°C for 1 h in the presence of inhibitors or vehicle (DMSO), followed by stimulation with fMLP. After fixation with 4 % paraformaldehyde for 5 min, cells were permeabilized with 80% acetone for 3 min and blocked with blocking buffer (10% fetal bovine serum, 0.05% w/v BSA in PBS) for 10 min. For experiments designed to detect phospho-proteins, methanol was used for permeabilization. Primary antibodies were diluted in blocking buffer as indicated, and incubated with cell samples overnight at 4°C. Secondary antibodies conjugated with AlexaFluor 488 or AlexaFluor 546 (1:500) in blocking buffer were added and incubated for 2 h in dark. In selected experiments, TRITC-phalloidin (1:1000) or Hoechst 33342 (1:1000) was added to stain F-actin or DNA, respectively. Coverslips were mounted onto glass slides and visualized with a Zeiss Axiovert fluorescent microscope. For data presentation, fluorescent intensities of each image were adjusted based on signals detected in neutrophil samples in the absence of primary antibodies.

Chapter 3: p21-Activated Kinases Regulate Cytoskeletal Reorganization and Directional Migration in Human Neutrophils

Asako Itakura, Joseph E. Aslan, Branden T. Kusanto, Kevin G. Phillips, Juliana E. Porter, Paul K. Newton, Xiaolin Nan, Robert H. Insall, Jonathan Chernoff, Owen J. T. McCarty

3.1 Abstract

Neutrophils serve as a first line of defense in innate immunity owing in part to their ability to rapidly migrate towards chemotactic factors derived from invading pathogens. As a migratory function, neutrophil chemotaxis is regulated by the Rho family of small GTPases. However, the mechanisms by which Rho GTPases orchestrate cytoskeletal dynamics in migrating neutrophils remain ill-defined. In this study, we characterized the role of p21-activated kinase (PAK) downstream of Rho GTPases in cytoskeletal remodeling and chemotactic processes of human neutrophils. We found that PAK activation occurred upon stimulation of neutrophils with formyl-Met-Leu-Phe (fMLP), and PAK accumulated at the actin-rich leading edge of stimulated neutrophils, suggesting a role for PAK in Rac-dependent actin remodeling. Treatment with the pharmacological PAK inhibitor, PF3758309, abrogated the integrity of RhoA-mediated actomyosin contractility and surface adhesion. Moreover, inhibition of PAK activity impaired neutrophil morphological polarization and directional migration under a gradient of fMLP, and was associated with dysregulated Ca^{2+} signaling. These results suggest that PAK serves as an important effector of Rho-family GTPases in neutrophil cytoskeletal reorganization, and plays a key role in driving efficient directional migration of human neutrophils.

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3.2 Introduction

Studies conducted in this chapter were designed to investigate the role of p21-activated kinase (PAK) in the regulation of cytoskeletal dynamics and chemotaxis of primary human neutrophils. Our immunofluorescence experiments demonstrate the activation and distinct subcellular localization pattern of PAK isoforms upon neutrophil stimulation with fMLP. By utilizing a pharmacological inhibitor of PAK, we show that PAK negatively regulates neutrophil adhesion and the "backness" polarity signal in response to fMLP, promoting efficient chemotaxis.

3.3 Background

Chemotaxis, the directed migration of cells driven by a gradient of external factors, is critical for the initial phases of innate immunity in which neutrophils sense chemoattractant mediators and migrate from the circulation through the endothelium to combat invading pathogens at sites of infection (Borregaard 2010). During chemotaxis, neutrophils undergo dramatic morphological changes as lamellipodia at the leading edge extend toward chemoattractant sources and a trailing edge forms at the neutrophil rear, termed the uropod. These polarized structures support the efficient migration of neutrophils at speeds of up to 30 μm/min (Bokoch 2005). Although many studies have described the molecular mechanisms that underlie the steps of chemotaxis (such as

directional sensing, polarization and motility), the manner in which neutrophil 'frontness' vs. 'backness' signals are coordinated to direct migration is still not fully understood.

Most chemoattractants, including the bacteria-derived chemotactic peptide formyl-Met-Leu-Phe (fMLP), bind to G protein-coupled receptors (GPCRs) expressed on the neutrophil surface. GPCR-ligand binding activates heterotrimeric G proteins and triggers various intracellular signaling pathways. The Rho family of GTPases, including Rac, Cdc42 and RhoA, have been shown to play key roles in the spatial and temporal regulation of neutrophil cytoskeletal remodeling downstream of chemoattractant receptors during chemotaxis (Bokoch 2005). In their activated forms, Rac and Cdc42 promote the extension and stabilization of an actin-rich leading edge at the front of neutrophils to generate a motile force, while active RhoA controls myosin II-dependent contractility and uropod retraction. Many signaling pathways have been shown to participate in a feedback loop that maintains the formation of a single leading edge and uropod. In the context of cytoskeletal rearrangement, the family of p21-activated kinases (PAKs) is a well-characterized target of Rac and Cdc42. To date, six isoforms of PAKs have been identified; PAK1, 2, 3 (Group I PAKs) and PAK 4, 5, 6 (Group II PAKs). Group I and II PAKs differ in their structural organizations and biochemical features including activation mechanisms (Baskaran *et al.* 2012). The binding of Rac or Cdc42 GTPases to the p21-binding domain (PBD) of group I PAKs induces autophosphorylation and activation of PAK as serine/threonine kinases, whereas the binding of Cdc42 to PBD does not serve to activate group II PAKs (Baskaran *et al.* 2012). In neutrophils, rapid phosphorylation of PAK1 and PAK2 isoforms has been observed after treatment with

various agonists (Knaus *et al.* 1995; Huang *et al.* 1998), and PAK1 has been found at the leading edge and phagocytic cup (Dharmawardhane *et al.* 1999). In a study using mouse neutrophils as well as non-myeloid (e.g. COS-7) and myeloid cell lines (e.g. HL-60 and RAW274), PAK1 induced Cdc42 activation by forming a complex with Gβγ and the guanine-nucleotide exchange factor (GEF) PIXα to promote actin polymerization and regulate PTEN distribution for efficient directional sensing (Li *et al.* 2003). However, the characterization of PAK function in human neutrophils has been hindered by the technical limitation that neutrophils are not susceptible to genetic manipulation in vitro, as they are terminally differentiated and have a short life span. Accordingly, studies of the functional roles of PAK in human neutrophils have been restricted to the use of gene transfection/knockdown strategies in leukemic cell lines. While PAK1- and PAK2-knockout mice have recently been established (Smith *et al.* 2008; Allen *et al.* 2009; Taglieri *et al.* 2011; Wang *et al.* 2011; Chow *et al.* 2012; Dorrance *et al.* 2013; Kosoff *et al.* 2013), the neutrophil phenotype in these mice has not yet been described.

In this study, we characterized the roles of the PAK signaling in relation to PI3K and Rho GTPase systems during fMLP-driven cytoskeletal reorganization in primary human neutrophils. Our data suggest that PAK2 is activated and accumulates to the neutrophil leading edge in response to fMLP to support Rac/Cdc42-mediated actin dynamics in a localized manner. In addition, PAK inhibition altered the subcellular localization of active RhoA and induced aberrant formation of vinculin-rich complexes. PAK kinase activity played a critical role in chemotaxis of human neutrophils as PAK inhibition led to a loss of directionality, increased spreading and decreased migration speed, whereas

Rac or PI3K inhibition resulted in impaired directionality or polarization, respectively. Taken together, these results suggest that PAKs establish 'frontness' signals by negatively regulating the surface adhesion and Rho-dependent 'backness' signals in human neutrophils, thus providing a mechanism for the crosstalk between Rho-family GTPases in neutrophil cytoskeletal dynamics and cell migration.

3.4 Materials and Methods

3.4.1 Reagents

For immunohistochemistry and western blot experiments, anti-αPAK (PAK1; sc-882), γPAK (PAK2; sc-373740), βPAK (PAK3; sc-1871) and PAK4 (sc-28779) were purchased from Santa Cruz (Dallas, TX, USA). Anti-PAK1/2/3 pThr423 (44-942G) was from Invitrogen (Grand Island, NY, USA). Anti-PAK2 pSer20 (2607) and myosin light chain2 pSer19 (3675) were from Cell Signaling (Boston, MA, USA). Anti-Rac1 (23A8) and Y27632 was from Millipore (Billerica, MA, USA). Anti-active Rac-GTP (26903), active RhoA-GTP (26904) and active-Cdc42-GTP (26905) antibodies were from NewEast Biosciences (King of Prussia, PA, USA). PF3758309 was prepared as previously described (Murray *et al.* 2010; Chow *et al.* 2012; Aslan *et al.* 2013).

3.4.2 Western blotting

After indicated treatments, human neutrophils were lysed in protein extraction reagent (#78505; Thermo Scientific, Waltham, MA, USA). Lysates were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% BSA for 1 h prior to incubation with primary antibodies overnight at 4°C. Following

washing with TBS-T, membranes were incubated with the appropriate secondary antibodies. Membranes were washed with TBS-T and developed using an enhanced-chemiluminescence system.

3.4.3 Immunofluorescence and total internal reflection fluorescence (TIRF) microscopy

For immunofluorescence microscopy, primary antibodies were used as follows: anti-PAK1/2/3 pThr423 (1:100), PAK2 pSer20 (1:50), Rac1 (1:100), active Rac-GTP (1:100), active RhoA-GTP (1:200), active Cdc42-GTP (1:100), myosin light chain2 pSer19 (1:200), vinculin (1:100), or actin (1:100). For TIRF microscopy, purified neutrophils were plated in 8-well chamber slide (Nunc) and stained for vinculin after the indicated treatments. Vinculin immunofluorescence in a focal section of the neutrophil within ~150 nm from the surface of coverslip was excited with a 488 nm-laser and detected via TIRF microscopy using a Nikon TE300 microscope equipped with a Nikon 60× oil immersion objective (NA=1.49) and an electron multiplied CCD camera. For data presentation, fluorescent intensities of each image were adjusted based on signals detected in neutrophil samples treated without primary antibody. Image analyses were performed using Colocalization Finder of ImageJ, or a custom program in MATLAB (The Mathworks, Inc., Natick, MA, USA).

3.4.4 Analysis of data

Data are shown as means ± SEM. For the quantification of immunofluorescence images, the Jarque-Bera test was used to evaluate normality of all parameters. One-way analysis of variance with Bonferonni post hoc correction was used to assess statistical significance

among parameters across multiple normally distributed cell parameters. The Kruskal-Wallis test was used to assess significance among non-normally distributed parameters. For the migration speed analysis, statistical analysis was performed using Student's t test. P-values of 0.05 or less were considered significant.

3.5 Results

3.5.1 PAK translocates to leading edge upon fMLP stimulation

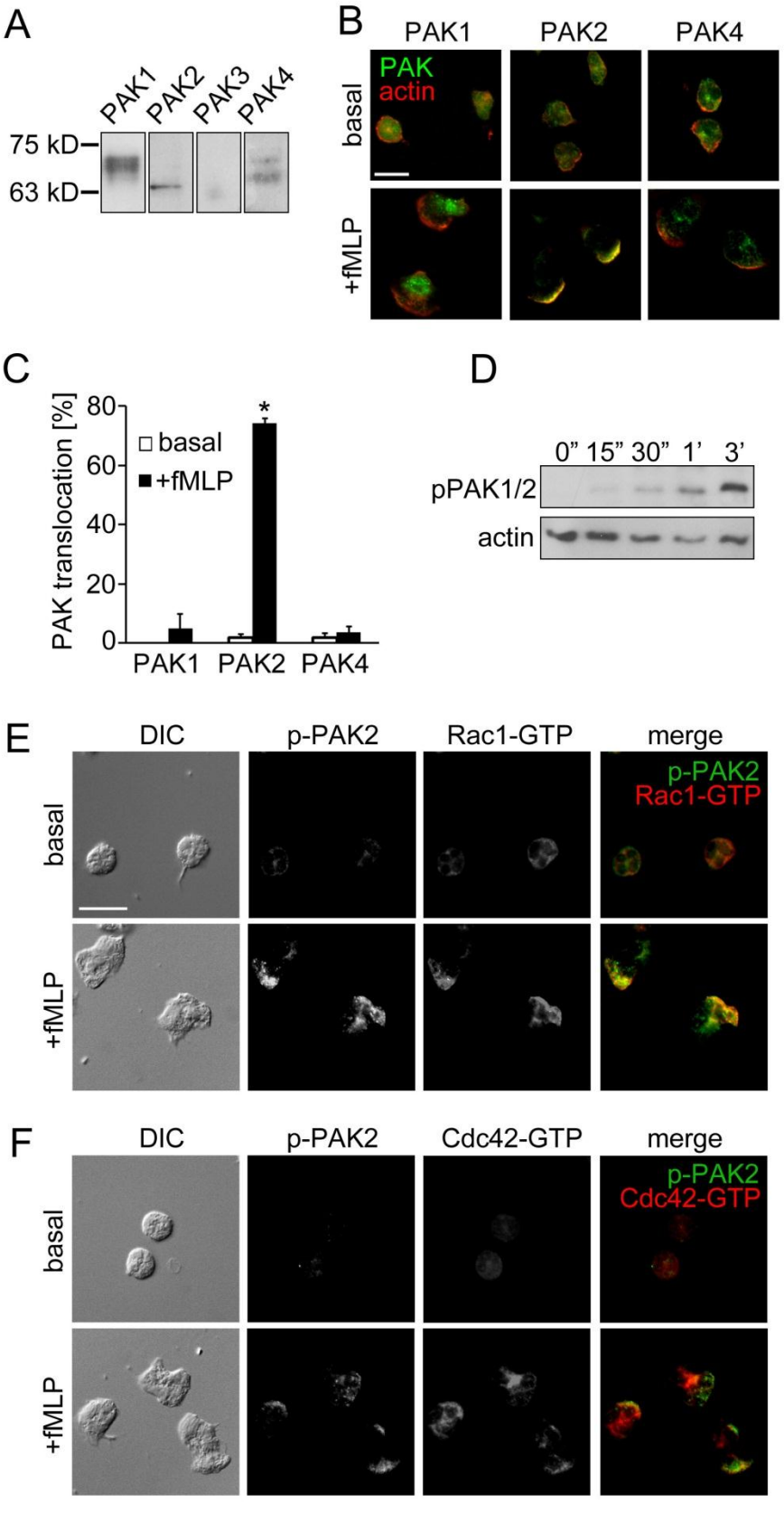
The PAK isoforms described in humans (PAK1-6) display a wide range of tissue distribution. PAK2 and PAK4 are ubiquitously expressed, while PAK1, PAK3, PAK5 and PAK6 expression are specific for tissues such as brain (PAK1, PAK3, PAK5 and PAK6) and spleen (PAK1) (Arias-Romero and Chernoff 2008; Kelly and Chernoff 2012). Previous studies of the PAK pathways in neutrophils have relied on biochemical assays to monitor PAK1 and PAK2 activation (Knaus *et al.* 1995; Huang *et al.* 1998). However, little is known about the expression and activity of PAK isoforms in regulating neutrophil cytoskeletal reorganization and migration. To characterize the expression of PAK isoforms in human neutrophils, whole human neutrophil lysates were separated by SDS-PAGE and examined for PAK expression by Western blot using PAK isoform-specific antibodies. As shown in Figure 3.1A, neutrophils express detectable levels of PAK1, PAK2 and PAK4. Next, to examine PAK isoform expression and subcellular localization, PAKs were studied by immunofluorescence microscopy of fixed neutrophils. PAK1, PAK2 and PAK4 were all detected in the cytosol of unstimulated neutrophils (Figure 3.1B). Upon the stimulation with the bacteria-derived peptide fMLP, PAK2 colocalized with the actin-rich leading edge of neutrophils, whereas PAK1 and PAK4 localization

was excluded from leading edge and primarily found in the cytosol and/or the back of stimulated cells (Figure 3.1B and 3.1C).

We next examined whether the translocation of PAK2 to the leading edge is associated with kinase activation downstream of fMLP receptor signaling. In primary human neutrophils, PAK undergoes rapid phosphorylation at specific Ser and Thr residues in response to a number of stimuli (Knaus *et al.* 1995; Huang *et al.* 1998). Phosphorylation of PAK1 Thr423 or PAK2 Thr402 is required for full catalytic function (Zenke *et al.* 1999; Zhao and Manser 2012), whereas phosphorylation at Ser20 of PAK2 regulates binding to the adaptor protein Nck to control PAK membrane localization as well as PAK kinase activity (Zhao *et al.* 2000; Bokoch 2003). The PAKs are activated upon the binding of the ~21 kD Rho GTPases Rac and Cdc42 (Zhao *et al.* 2000; Bokoch 2003). Along these lines, Rac and Cdc42 GTPases promote lamellipodia formation and regulate lamellipodia stability through a positive feedback loop at the actin-rich leading edge of migrating cells (Raftopoulou and Hall 2004; Heasman and Ridley 2008), including neutrophils (Weiner *et al.* 2002; Srinivasan *et al.* 2003; Bokoch 2005). Our Western blot analysis showed that PAK1 Thr423 and PAK2 Thr402 residues were phosphorylated within 3 min after neutrophil stimulation with fMLP (Figure 3.1D). In parallel, phosphorylated PAK2 Ser20 accumulated at the neutrophil leading edge and colocalized with active, GTP-bound Rac1 (71.7 ± 4.4% colocalization; Figure 3.1E) and Cdc42 (49.8 ± 6.0% colocalization; Figure 3.1F). In parallel, Rac2, a Rac isoform expressed in myeloid cells, was found to localize to the cytosol and toward the leading edge with 63.6 ± 8.4% of the immunofluorescence signal of Rac2 colocalizing with Rac1, following

neutrophil stimulation with fMLP for 3 min. Together, these results suggest that a coupled activation of Rac/Cdc42 and PAK2 are involved in leading edge formation during neutrophil migration.

Next, we investigated the mechanisms that regulate PAK activation in lamellipodia dynamics of activated neutrophils. To detect the activation of PAK, we performed immunofluorescence microscopy experiments using primary antibodies specific for phosphorylated PAK1/2 Thr423/402. Fluorescence microscopy revealed Threonine-phosphorylated PAK1/2 in neutrophils upon stimulation with fMLP, which localized to the actin-rich leading edge (Figure 3.1G). To test the role of Rac GTPase in neutrophil PAK activation, we next analyzed the effect of Rac inhibition on PAK activation and neutrophil morphology using the Rac1/2 inhibitor EHT1864. In Rac-inhibited neutrophils, phosphorylated PAK1/2 Thr423/402 remained at the leading edge (Figure 3.1G and 3.1H). In neutrophils, Rac activation is in part regulated by PI3Ks which promote the localization of Rac-activating GEFs to the neutrophil leading edge (Han *et al.* 1998; Welch *et al.* 2002; Zhao *et al.* 2007). Accordingly, an impairment of PAK2 activation has been reported downstream of RANTES-induced chemotaxis signaling in PI3Kγ-deficient mouse macrophages (Weiss-Haljiti *et al.* 2004). As shown in Figure 3.1G and 3.1H, treatment of neutrophils with the PI3K inhibitor, wortmannin, failed to block PAK phosphorylation in response to fMLP, but led to a non-polarized accumulation of actin and phospho-PAK. This result suggests that like Rac, PI3K activity may also play a role in the intracellular trafficking of active PAK, but not directly in PAK activation.


A
PAK1
PAK2
PAK3
PAK4
75 kD
63 kD
B
PAK1
PAK2
PAK4
PAK
actin
basal
+fMLP
C
PAK translocation [%]
80
60
40
20
0
basal
+fMLP
*
PAK1
PAK2
PAK4
D
0" 15" 30" 1' 3'
pPAK1/2
actin
E
DIC
p-PAK2
Rac1-GTP
merge
p-PAK2
Rac1-GTP
basal
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DIC
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basal
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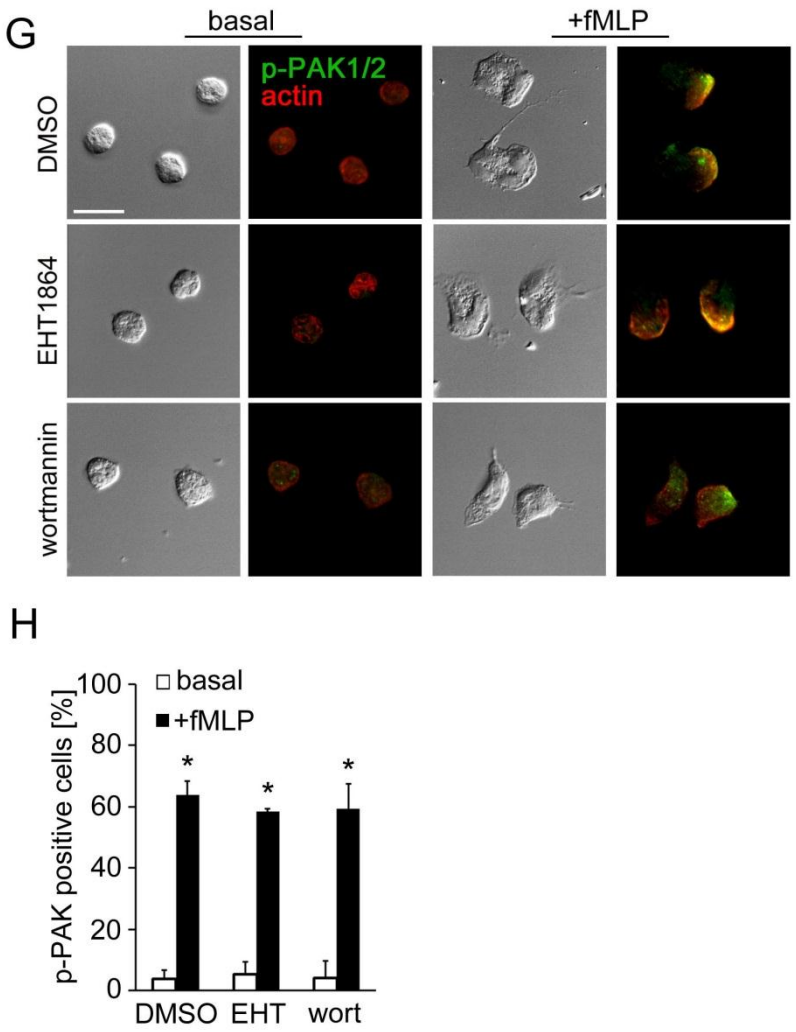
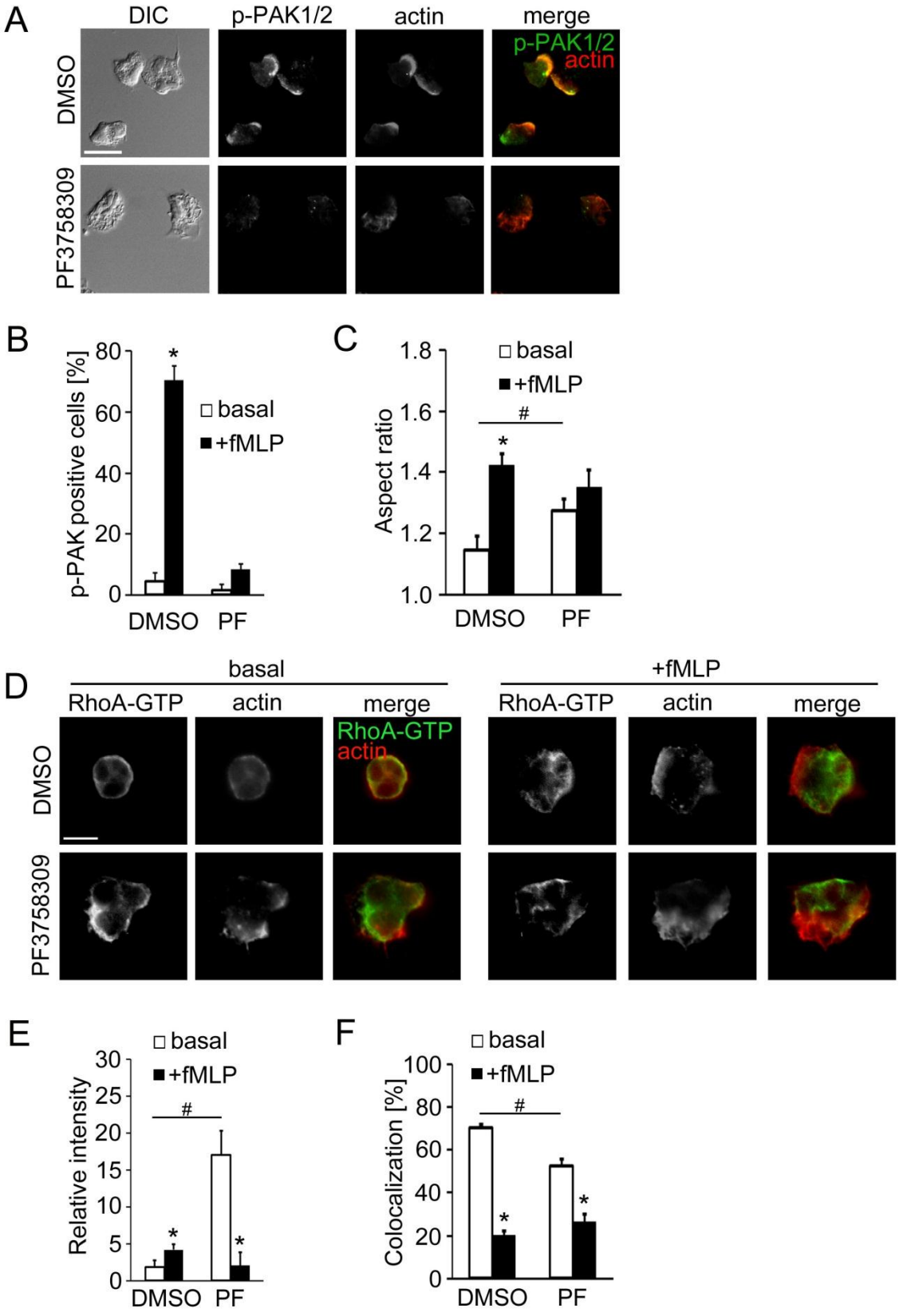


Figure 3.1 PAK2 localizes to the leading edge of activated neutrophils.

Replicate samples of total neutrophil (PMN) cell lysates (50 μg per lane) were analyzed for (A) the expression of PAK1 (68 kD), PAK2 (61 kD), PAK3 (65 kD) and PAK4 (72 kD) or (D) phosphorylation of PAK1/2 Thr432/402 at indicated time points after the addition of fMLP (10 nM) by Western blot. Human neutrophils adherent on fibronectin surfaces were treated in the presence or absence of fMLP (10 nM) for 3 min and stained for (B) PAK1, PAK2 or PAK4 (green) and F-actin (red), (E) phospho-PAK2 Ser20 (green) and Rac1-GTP (red), (F) phospho-PAK2 Ser20 (green) and Cdc42-GTP (red), or (G) phospho-PAK1/2 Thr423/402 (green) and F-actin (red) . In selected experiments, neutrophils were pretreated with vehicle (0.1% DMSO), EHT1864 (Rac1/2 inhibitor, 50 μM) or wortmannin (PI3K inhibitor, 100 nM). Results are quantified from at least 60 cells and presented as the mean percentage ± SEM of (C) neutrophils displaying PAK immunofluorescence at actin-rich leading edge, or (H) neutrophils displaying phospho-PAK1/2 immunofluorescence in the fields of view. Representative images obtained from 3 independent experiments are shown. * $P < 0.05$, compared to the basal level.
Scale bar = 10 μm

3.5.2 PAK activation and Rho GTPases mediate neutrophil backness signals

Neutrophil morphological polarity is maintained through a balance of 'frontness' and 'backness' signals which have been shown to be mediated by the Rho GTPases Rac/Cdc42 GTPases and RhoA, respectively (Bokoch 2005). The putative roles of the Rho GTPases and PAK in leading edge dynamics led us to hypothesize that PAKs may maintain neutrophil 'frontness' by modulating 'backness' during neutrophil polarization. To test this hypothesis, we assessed the effects of PAK inhibition on RhoA GTPase activation and localization using the pharmacological PAK inhibitor, PF3758309 (Murray *et al.* 2010; Chow *et al.* 2012; Aslan *et al.* 2013), which inhibited the phosphorylation of PAK1/2 Thr423/402 (Figure 3.2A and 3.2B) in fMLP-stimulated neutrophils. While neutrophils developed a morphological polarity following fMLP stimulation, characterized by a significant increase in aspect ratio (a function of the largest cell diameter and the smallest diameter), PF3758309-treated cells underwent polarization in the absence of any stimulation (Figure 3.2C). In vehicle-treated cells, fMLP-induced polarization was associated with an increase in the immunofluorescence of active RhoA-GTP (Figure 3.2D and 3.2E) and a decrease in the colocalization of RhoA-GTP with actin (Figure 3.2D and 3.2F), suggesting that fMLP induced active RhoA accumulation at the uropod. In contrast, PF3758309-treated cells displayed similar signatures of cell polarization in the absence of fMLP with a significantly higher level of active RhoA as compared to control cells (Figure 3.2D-F). Stimulation of PAK-inhibited cells with fMLP resulted in a comparable level of active RhoA to fMLP-stimulated control cells (Figure 3.2D-F). These results suggest that PAK contributes to neutrophil cytoskeletal dynamics under basal conditions by suppressing RhoA-GTP accumulation and relocalization.


A
DIC
p-PAK1/2
actin
merge
p-PAK1/2
actin
DMSO
PF3758309
B
p-PAK positive cells [%]
80
60
40
20
0
*
□ basal
■ +fMLP
DMSO
PF
C
Aspect ratio
1.8
1.6
1.4
1.2
1.0
□ basal
■ +fMLP
#
*
DMSO
PF
D
basal
+fMLP
RhoA-GTP
actin
merge
RhoA-GTP
actin
DMSO
PF3758309
E
Relative intensity
30
25
20
15
10
5
0
□ basal
■ +fMLP
#
*
*
DMSO
PF
F
Colocalization [%]
100
80
60
40
20
0
□ basal
■ +fMLP
#
*
*
DMSO
PF

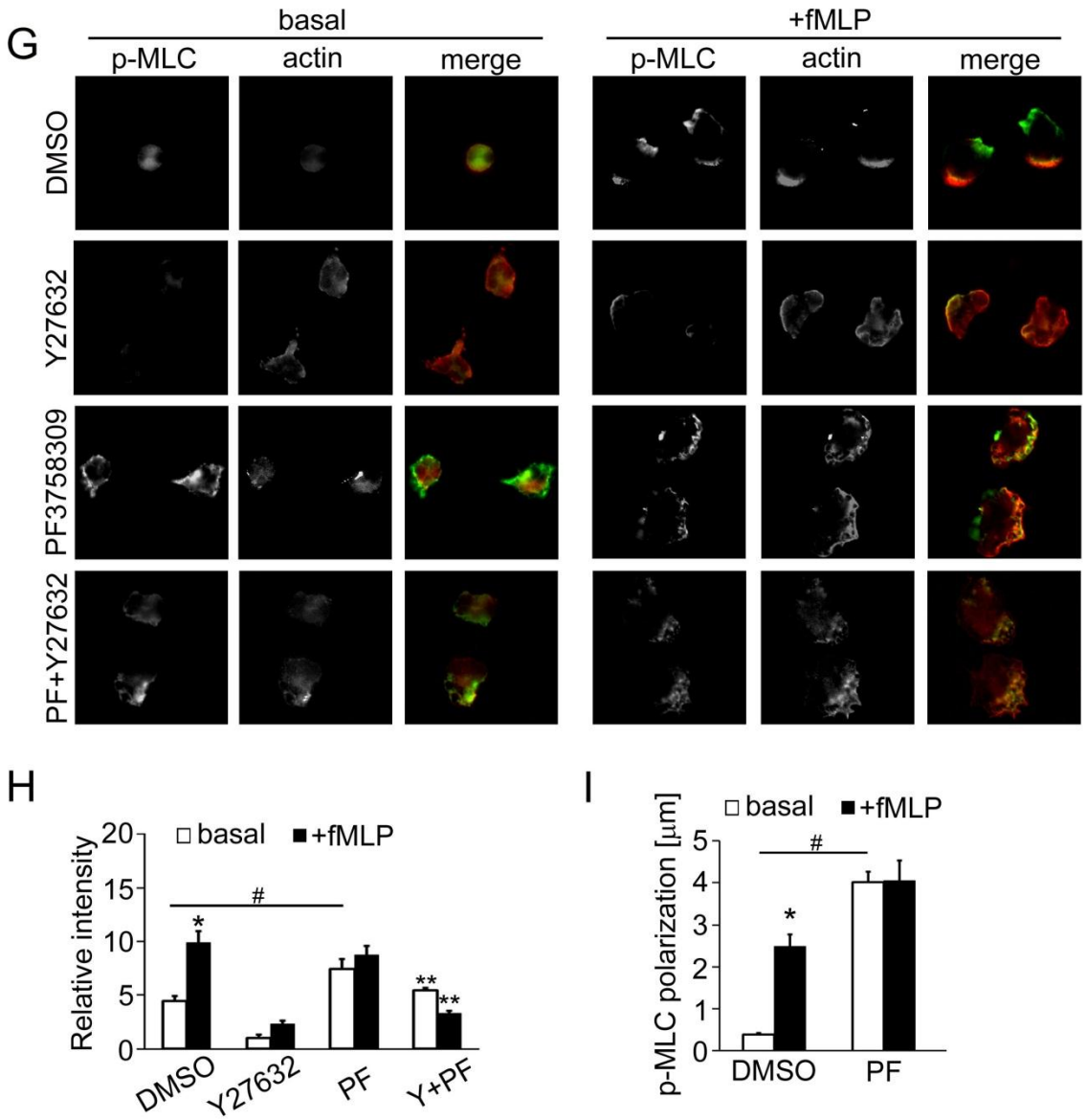


Figure 3.2 PAK inhibition leads to an accumulation of active RhoA and phosphorylated myosin light chain.

(A) Human neutrophils adherent on fibronectin surfaces were pretreated with vehicle (0.1% DMSO) or PF3758309 (PF; PAK inhibitor, 10 µM), and stimulated with fMLP (10 nM) for 3 min and stained for phospho-PAK1/2 Thr423/402 (green) and F-actin (red). (B) The mean percentage of cells displaying phospho-PAK1/2 immunofluorescence at basal level (white bars) or after stimulation (black bars) was quantified from at least 50 cells per treatment. (C) Cell circularity was analyzed using MATLAB and presented as aspect ratio of individual neutrophils at basal level (white bars) or after fMLP stimulation (black bars). (D) Neutrophils were pretreated as indicated in (A) and stimulated with fMLP (10 nM). Cells were stained for active RhoA-GTP (green) and F-actin (red). (E) The mean relative intensity of RhoA-GTP immunofluorescence or (F) the mean percentage of RhoA-GTP/actin colocalization of individual cell area at basal level (white bars) or after fMLP stimulation (black bars). (G) Neutrophils were pretreated with the inhibitors indicated in (A), or with Y27632 (ROCK inhibitor, 10 µM), or a combination of 10 µM PF3758309 and 10 µM Y27632 (PF+Y27632) and stained for phospho-myosin light chain (p-

MLC; green) and F-actin (red). (H) Relative p-MLC fluorescence intensity or (I) the distance between DIC cell centroid and p-MLC fluorescence centroid at basal level (white bars) or after fMLP stimulation (black bars). Representative images obtained from at least 3 independent experiments are shown. * $P< 0.05$ compared to the basal level; # $P< 0.05$, compared to DMSO-treated cells; ** $P< 0.05$, compared to cells treated with Y27632 alone. Scale bar = (A) and (G) 5 μm; (D) 10 μm.

Active RhoA binds to and activates a number of downstream effectors including the Rho-associated kinase ROCK (Bokoch 2005). The RhoA-ROCK axis has been shown to be essential for myosin II-mediated actomyosin contraction in various cell types (Heasman and Ridley 2008) and for surface detachment of migrating leukocytes (Alblas *et al.* 2001), in which ROCK phosphorylates myosin light chain (MLC) at Ser19 to promote the actin-stimulated ATPase activity of myosin. In neutrophils, MLC phosphorylation plays a key role in uropod retraction (Eddy *et al.* 2000), and in the maintenance of backness polarity (Xu *et al.* 2003). To test whether PAK activity has a role in actomyosin contraction, we next examined the subcellular distribution of phospho-MLC under basal and PAK-inhibited conditions. fMLP induced an increase in phospho-MLC (Figure 3.2G and 3.2H) at sites distant from cell centroids (Figure 3.2I), suggesting a polarized mechanism of actomyosin contraction. Phospho-MLC immunofluorescence was abrogated in the presence of the ROCK inhibitor, Y27632 (Figure 3.2G and 3.2H). Inhibition of PAK with PF3758309 led to an accumulation of phospho-MLC in unstimulated and stimulated cells (Figure 3.2G and 3.2H). Interestingly, the loss of MLC phosphorylation resultant from Y27632 was partially recovered by co-treatment of PF3758309 with Y27632 (Figure 3.2G and 3.2H). Taken together, this data suggests that PAK regulates actomyosin contraction in migrating neutrophils via ROCK-dependent and -independent pathways.

3.5.3 PAK negatively regulates focal complex assembly and surface adhesion

To stabilize the lamellipodium, small adhesion structures, known as focal complexes, are formed at the leading edge via Rac/Cdc42 activity which link integrins to surface and cytoskeletal proteins (Ridley *et al.* 2003). To determine the contribution of PAK activity to focal complex assembly in neutrophils, we examined the localization of vinculin, a marker of focal complex assembly, under control and pharmacologically-inhibited conditions. As seen in Figure 3.3A and 3.3B, vinculin localized to the cytosol of unstimulated neutrophils and accumulated at the actin-rich leading edge as well as the uropod after fMLP stimulation, indicating the presence of vinculin-containing cell adhesions. Neutrophils treated with the Rac1/2 inhibitor EHT1864 displayed a similar pattern of vinculin localization as compared to control cells, whereas treatment with the PI3K inhibitor wortmannin led to the less polarized localization of vinculin immunofluorescence (Figure 3.3A and 3.3B). Interestingly, PAK-inhibited neutrophils formed vinculin-positive, filopodia-like structures at the cell periphery in response to fMLP (Figure 3.3A). Similar vinculin clusters have been found in neutrophils treated with TNF-α (Lokuta and Huttenlocher 2005), which confers inhibitory signals to neutrophil motility by promoting firm adhesion and limiting polarization. Accordingly, we next aimed to examine whether PAK inhibition enhanced neutrophil adhesion strength by increasing surface contacts. Neutrophil adhesion complexes were studied by total internal reflection fluorescence (TIRF) microscopy, by which immunofluorescence signals adjacent (~150 nm) to the interface between a surface and a specimen are exclusively detected with a high spatial resolution as an indicator of surface contact area. As seen in Figure 3.3C and 3.3D, TIRF microscopy for vinculin immunofluorescence

revealed that PF3758309-treatment dramatically enhanced the level of vinculin-mediated surface contact as compared to vehicle, both in the presence and absence of fMLP. Together, these results demonstrate that PAK may negatively regulate surface adhesion during neutrophil polarization.

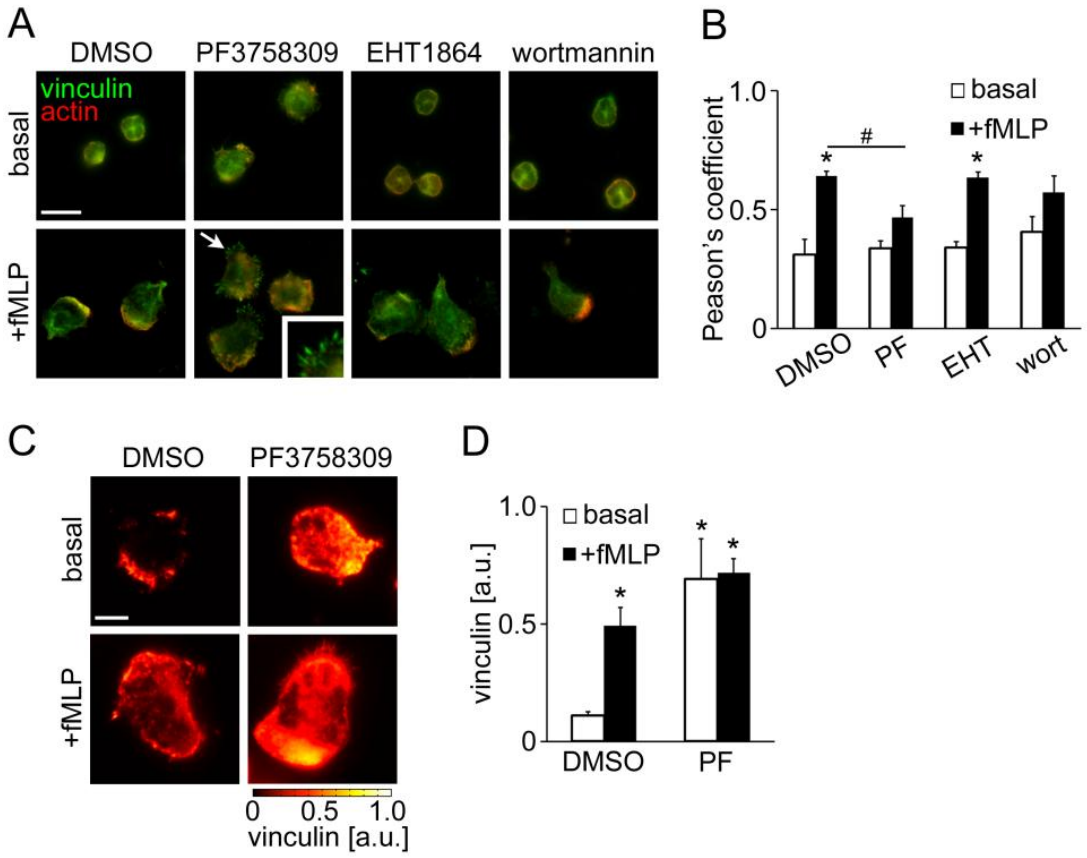


Figure 3.3 PAK inhibition enhances vinculin-mediated surface contacts.

Human neutrophils adherent on fibronectin surfaces were treated in the presence or absence of fMLP (10 nM) for 3 min. In selected experiments, neutrophils were pretreated with PF3758309 (PF; PAK inhibitor, 10 μM), EHT1864 (Rac1/2 inhibitor, 50 μM) or wortmannin (PI3K inhibitor, 100 nM). (A) Cells were stained for vinculin (green) and F-actin (red). Arrows indicate filopodia-like vinculin clusters shown in the insets. (B) Pearson's coefficient for vinculin and actin immunofluorescence in cells treated as indicated in (A). (C) Neutrophil surface vinculin was visualized using TIRF microscopy. Arbitrary units (a.u.) for vinculin signal intensity are shown. (D) The mean TIRF signals of vinculin immunofluorescence were quantified from at least 5 cells per treatment. * P< 0.05 compared to the basal vinculin signal in DMSO-treated cells.
Scale bar = (A) 10 μm; (C) 2 μm.

3.5.4 PAK plays a role in neutrophil directional migration and intracellular Ca^{2+} release

The above described PAK-mediated regulation of actomyosin contractility and surface adhesion led us to further examine the role of PAK in neutrophil chemotaxis. The determining components of efficient chemotaxis include directional sensing, morphological polarization and motility. Our data show that upon exposure to an fMLP gradient, neutrophils first uniformly spread and then immediately developed morphological polarity, in which the leading edge formed distinct lamellipodia to differentiate from the uropod (Figure 3.4E). Within 20 min, neutrophils migrated toward the fMLP gradient at the average speed of 7.2 ± 0.5 μm/min (Figure 3.4A and 3.4B).

To better understand the roles of PI3K, Rho GTPases and PAK signaling pathways downstream of fMLP that regulate neutrophil chemotaxis, we characterized the directionality, polarity and motility of migrating neutrophils in the presence of pharmacological inhibitors. Neutrophil directionality was partially disrupted in the presence of the PI3K inhibitor, wortmannin (Figure 3.4A), although migration speed was unaffected (6.2 ± 0.85 μm/min; Figure 3.4B). The presence of the Rac1/2 inhibitor, EHT1864, led to a random migration pattern with a migration speed comparable to vehicle-treated cells (6.3 ± 0.56 μm/min; Figure 3.4A and 3.4B). Treatment of neutrophils with the ROCK inhibitor, Y27632, did not affect the directionality of chemotaxis or significantly reduced migration speed (5.1 ± 0.47 μm/min; Figure 3.4A and 3.4B). In contrast, neutrophils treated with PF3758309 underwent enhanced spreading in response to fMLP (Figure 3.4E) and randomly migrated at a dramatically

reduced migration speed (3.1 ± 0.21 μm/min; Figure 3.4A, 3.4B and 3.4C) in a concentration-dependent manner (Figure 3.4D). These results suggest that PAK activity plays a crucial role in coordinating directional sensing, polarity and motility of chemotaxing neutrophils.

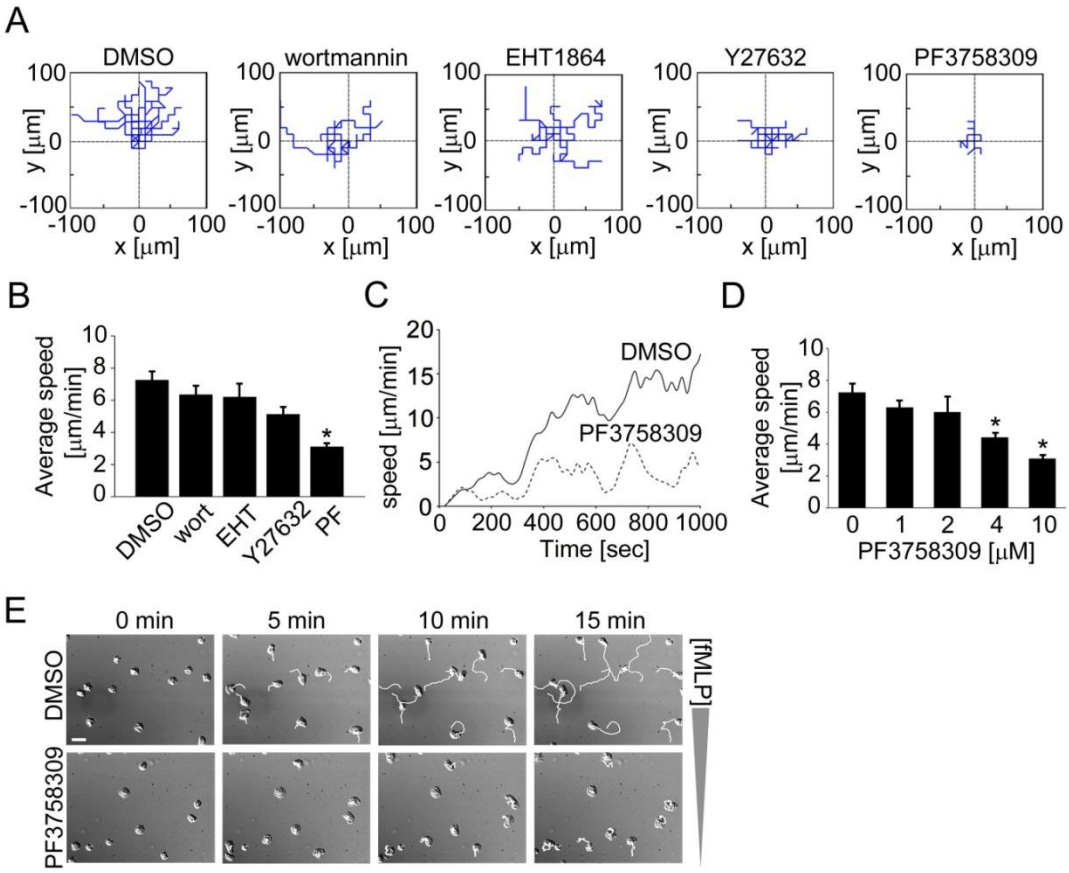


Figure 3.4 PAK inhibition blocks neutrophil chemotaxis.

Human neutrophils were treated with vehicle (0.1% DMSO), EHT1864 (Rac1/2 inhibitor, 50 μM), wortmannin (PI3K inhibitor, 100 nM), Y27632 (ROCK inhibitor, 10 μM), or PF3758309 (PAK inhibitor, 10 μM) for 15 min and chemotaxis was induced by adding fMLP (10 nM) in the outer well of an Insall chamber. Time-lapse images were obtained every 10 s for 20 min at 40× magnification using DIC microscopy. (A) The migration paths of individual cells were quantified as spider plots using MtrackJ (ImageJ) and MATLAB. (B) Average migration speeds of neutrophils treated as indicated in (A). (C) Representative time-lapse images of DMSO- or PF3758309-treated cells at the first 0, 5, 10, 15 min with cell paths (white). (D) Representative kinetics of cell migration was plotted against time. (E) A dose-dependent inhibitory effect of PF3758309 on average migration speed. Results were obtained from 3 independent experiments and data quantification was performed for at least 30 cells per treatment. * $P < 0.05$ compared to the average migration speed of DMSO-treated cells. Scale bar = 20 μm.

fMLP receptor signaling induces intracellular Ca^{2+} transients via the activation of the Gβγ complex and phospholipase C, triggering Ca^{2+}-dependent downstream pathways such as protein kinase C (Li *et al.* 2000; Bokoch 2005). These pathways have been shown to collectively play a role in cytoskeletal dynamics in neutrophils (Liu *et al.* 2010). PAK1-deficient bone marrow-derived mast cells have been shown to exhibit diminished intracellular calcium mobilization (Allen *et al.* 2009; Kosoff *et al.* 2013). However, a role for the PAK pathway in the coupling of calcium signaling during neutrophil chemotaxis has not been explored. Next, to characterize the role of PAK in intracellular Ca^{2+} mobilization, neutrophils were loaded with the Ca^{2+}-sensitive reporter dye fluo-4 AM and allowed to migrate under a fMLP gradient while intracellular Ca^{2+} release was measured for 15 min. As shown in Figure 3.5, neutrophils exhibited a single spike of Ca^{2+} release upon exposure to fMLP, followed by a gradually decreasing level of Ca^{2+} during chemotaxis. In contrast, neutrophils treated with the PAK inhibitor, PF3758309, displayed multiple peaks of Ca^{2+} release and failed to develop polarized morphology or migrate toward the fMLP gradient (Figure 3.5). These data imply that PAK activity contributes to the regulation of intracellular Ca^{2+} transients during neutrophil chemotaxis.

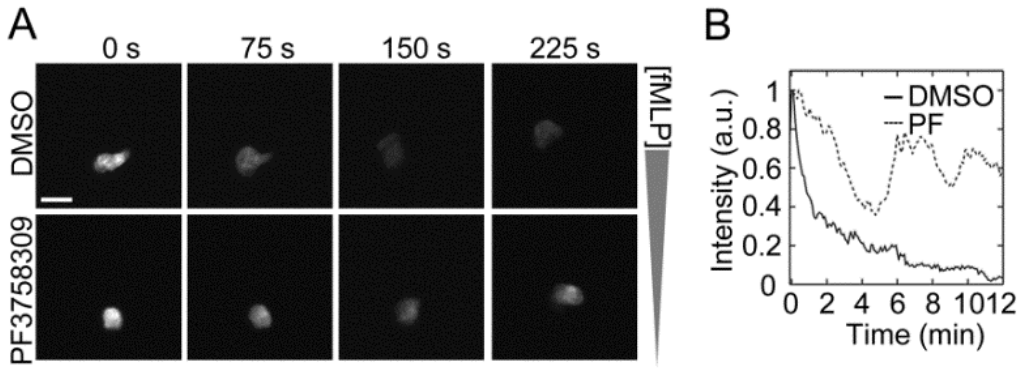


Figure 3.5 PAK inhibition alters neutrophil calcium signaling.

Human neutrophils were loaded with the intracellular Ca^{2+} dye fluo-4 (2 μM) and plated on fibronectin-coated surfaces. After treatment with DMSO or PF3758309 (10 μM), neutrophil chemotaxis was induced by the addition of fMLP in an Insall chamber. Intracellular Ca^{2+} release was monitored every 5 s for 15 min by fluorescent microscopy. (A) Time-lapse images after the peak Ca^{2+} spike were shown. (B) Ca^{2+} spikes in cells treated with 0.1% DMSO (black line) or PF3758309 (PF; PAK inhibitor, dotted line) were quantified and presented as mean intensity of at least 5 neutrophils in a field of view. Data shown is representative from 3 independent experiments. Scale bar = 20 μm.

3.6 Discussion

The efficient migratory ability of neutrophils requires a network of interactions between distinct cytoskeletal signaling systems, namely frontness signals regulated by actin-dependent membrane protrusion and backness signals mediated by myosin-dependent cell contraction. Recent studies suggest that crosstalk processes between frontness and backness modules during the initiation, establishment and maintenance of neutrophil polarization are dynamic and complex (Ku *et al.* 2012). Moreover, the mediators of temporal and spatial regulation in this crosstalk network remain to be elucidated. In this study, we identified the p21-activated kinase PAK as a regulator of neutrophil cytoskeletal reorganization. The PAK family proteins serve as effectors downstream of Rac and Cdc42 GTPases and are noted for roles in cytoskeletal dynamics, gene transcription, survival signaling, and cell cycle progression (Bokoch 2003; Zhao and

Manser 2012). Here we find that the kinase activity of PAK is required for the efficient directional migration of human neutrophils, where PAK2 may serve key roles in frontness signals at the leading edge. The absence of PAK activity resulted in an impairment of polarization, which was associated with increased backness signals and surface adhesions. Together, as depicted in Figure 3.6, our data describe a differential spatial regulation of PAK isoforms in fMLP-stimulated human neutrophils and highlights a potential mechanism for PAK-mediated crosstalk between Rho GTPases during cytoskeletal reorganization.

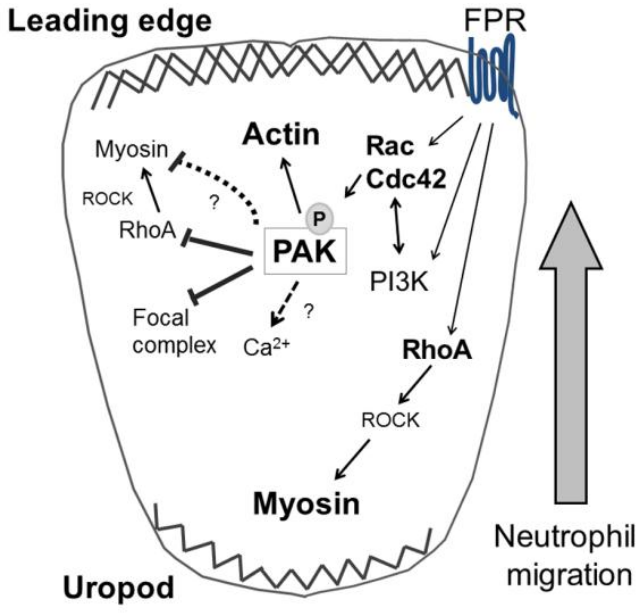


Figure 3.6 A model for PAK-mediated cytoskeletal regulation in human neutrophils.
PAK coordinates the crosstalk between Rho GTPases and cytoskeletal dynamics in migrating neutrophils.

The expression of the six PAK isoforms differs according to cell and tissue type, implying that PAK family isoforms may have specific and distinct functions in differing physiological contexts (Arias-Romero and Chernoff 2008; Kelly and Chernoff 2012).

Recent studies have reported functional differences for PAK1 and PAK2 in various model systems. In hematopoietic stem and progenitor cells, PAK2 plays a major role in coordinating cytoskeletal and proliferative pathways that are essential for engraftment of these cells (Dorrance *et al.* 2013). PAK1 and PAK2 have been shown to differentially modulate adhesion, RhoA activity and MLC phosphorylation during mast cell degranulation (Kosoff *et al.* 2013) and tumor cell migration (Coniglio *et al.* 2008). Here, we find that human neutrophils express PAK1, PAK2 and PAK4, and that PAK2 may play a specific role in the regulation of actin dynamics at the leading edge upon neutrophil activation. Whether PAK1 and PAK4 have any role in mediating cytoskeletal regulation at non-leading edge regions of migrating neutrophil requires further investigation. Along these lines, PAK1, but not PAK2, has been shown to positively regulate IgE-mediated degranulation in mast cells by regulating extracellular Ca^{2+} influx through a mechanism involving cytoskeletal dynamics, although the molecular details of such events remain to be explored (Allen *et al.* 2009; Kosoff *et al.* 2013). It is possible that the dysregulation of Ca^{2+} mobilization by PAK inhibition in our human neutrophil study was a direct result of the blockade of the activity of specific PAK isoforms. Further studies are required to determine whether any of these PAK isoforms can serve as a scaffold for signaling proteins to facilitate polarization, dependently or independently of their kinase activity, in human neutrophils. Notably, the pharmacological PAK inhibitor PF3758309 utilized in this study does not allow for the selective inhibition of specific PAK isoforms, as this compound inhibits all PAK isoforms (PAK1-6) (Chow *et al.* 2012). Future studies will aim to define specific roles for PAK isoforms in neutrophil function

using bone-marrow specific, inducible PAK-deficient mouse models, together with second generation PAK inhibitors with distinct target specificities.

Antagonistic signals between Rac1/2, Cdc42 and RhoA stabilize the polarity of migrating cells by restricting actin polymerization to the leading edge and myosin-dependent contraction to the uropod (Gardiner *et al.* 2002; Bokoch 2005). For efficient uropod retraction during migration, the RhoA-ROCK-myosin axis plays an essential role in myeloid cell lines, as the expression of dominant negative RhoA (Xu *et al.* 2003) or the pharmacological inhibition of ROCK (Worthylake and Burridge 2003; Xu *et al.* 2003) leads to multiple lamellipodial protrusions and a mislocalization of phosphorylated MLC. In our experiments, the inhibition of PAK in unstimulated neutrophils promoted the accumulation of active RhoA and phospho-MLC at the cell periphery (Figure 3.2), suggesting that the basal PAK activity can suppress the development of backness signals. The molecular mechanisms that bridge PAK activation and RhoA regulation in neutrophils have not been completely described. As Rho GTPases can be activated by multiple GEFs, which promote the release of GDP and the binding of GTP in specific cellular contexts (Heasman and Ridley 2008), it is possible that PAK may modulate the activity of Rho GEFs such as GEFH1 (Zenke *et al.* 2004) and PDZ RhoGEF (Barac *et al.* 2004) in a localized manner. Furthermore, our data imply that PAK can regulate MLC phosphorylation through RhoA/ROCK-independent pathways (Figure 3.2G and 3.2H). Potential alternative pathways for MLC phosphorylation include Ca^{2+}-dependent myosin light chain kinase (MLCK), as the phosphorylation of MLCK by PAK has been shown to inhibit MLCK activity (Sanders *et al.* 1999).

In motile cells, adhesion strength is one of the determining factors for migratory ability. Focal complexes, consisting of integrins and adhesion complex proteins such as vinculin, stabilize the lamellipodium by supporting surface attachment, and contribute to efficient cell migration (Ridley *et al.* 2003). Several studies have shown that in transfected cell lines, the binding of active Rac/Cdc42 to PAK induces the recruitment of PAK to focal complexes (Manser *et al.* 1997; Sells *et al.* 1997). A study by Delorme-Walker et al. utilizing an epithelial cell line demonstrated that PAK inhibition resulted in the disruption of actin turnover, myosin II and focal adhesion dynamics (Delorme-Walker *et al.* 2011). In rapidly migrating cells such as neutrophils, however, the formation of focal complexes is less visible, likely as a result of their rapid migration (Ridley *et al.* 2003). Our current study demonstrated that the absence of PAK activity led to a filopodia-like clustering of vinculin in human neutrophils (Figure 3.3). These results provide a novel regulatory mechanism for PAK-dependent surface adhesion in migrating neutrophils.

In summary, our data describe PAK as a key coordinator of cytoskeletal dynamics during cytoskeletal reorganization in human neutrophils and demonstrate that PAK mediates the Rho GTPase crosstalk to maintain the balance of front and back polarity signals. Future studies are required to characterize the functional roles of PAK in neutrophil inflammatory responses such as oxidative burst, degranulation, phagocytosis and neutrophil extracellular trap formation.

Chapter 4: Pivotal Role for the mTOR Pathway in the Formation of Neutrophil Extracellular Traps (NETs) via Regulation of Autophagy

Asako Itakura, Owen J. T. McCarty

4.1 Abstract

Autophagy is an essential cellular mechanism for cell homeostasis and survival, by which damaged cellular proteins are sequestered in autophagosomal vesicles and cleared through lysosomal machinery. The autophagy pathway also plays an important role in immunity and inflammation via pathogen clearance mechanisms mediated by immune cells, including macrophages and neutrophils. In particular, recent studies have revealed that autophagic activity is required for the release of neutrophil extracellular traps (NETs), representing a distinct form of active neutrophil death, namely NETosis. Although NETs formation is beneficial during host defense against invading pathogens, the mechanisms that promote excessive NETosis under pathological conditions remain ill-defined. In the present study, we aimed to characterize the role of the mammalian target of rapamycin (mTOR) in NETosis. As mTOR kinase is known as a key regulator of autophagy in many mammalian cells including neutrophils, we hypothesized that mTOR may play a regulatory role in NETs release by regulating autophagic activity. Our data show that the pharmacological inhibition of the mTOR pathway accelerated the rate of NETs release following neutrophil stimulation with the bacteria-derived peptide formyl-Met-Leu-Phe (fMLP), while autophagosome formation was enhanced by mTOR inhibitors. This increased mTOR-dependent NETs release was sensitive to inhibition of respiratory burst or blockade of cytoskeletal dynamics. Overall, this study demonstrates a pivotal role for

the mTOR pathway in coordinating intracellular signaling events downstream of neutrophil activation leading to NETosis.

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4.2 Introduction

Studies in Chapter 3 characterize the signaling pathways that regulate cytoskeletal reorganization during neutrophil chemotaxis driven by the chemoattractant fMLP. Upon their arrival at the sites of infection via chemotaxis, neutrophils utilize multiple strategies to kill microorganisms, including the release of NETs. However, it is still unknown what cellular events drive NETosis over other microbicidal functions in response to the end-target stimuli such as fMLP. In Chapter 4, studies describe the pivotal role of the mammalian target of rapamycin (mTOR) pathway in NETosis by controlling autophagic activity downstream of fMLP signaling.

4.3 Background

Autophagy plays a primary role in cellular protein homeostasis by regulating turnover of stable and damaged intracellular proteins (Ravikumar *et al.* 2010). During autophagy, a double-membrane vesicle containing captured cellular components develops into an autophagosome, followed by fusion with a lysosome to break down the vesicle contents and inner membrane. One of the key regulators of autophagy includes mammalian target of rapamycin (mTOR), a serine/threonine kinase that regulates cell growth, proliferation and protein synthesis (Mehrpour *et al.* 2010; Mizushima 2010; Levine *et al.* 2011). In

mammalian cells, mTOR kinase directly interacts with the unc-51 like kinase (ULK) complex, which plays a major role in controlling early steps of autophagosome formation, to inhibit membrane targeting of the ULK complex and other autophagy-related gene (Atg) proteins (Mehrpour *et al.* 2010; Mizushima 2010). Inhibition of mTOR activity is known to play an essential step in the initiation of autophagy.

Autophagy has been shown to play a key role in regulating leukocyte responses ranging from phagocytosis of pathogens (Levine *et al.* 2011) and cytokine secretion (Jones *et al.* 2013) to the formation of neutrophil extracellular traps (NETs) (Remijsen *et al.* 2011). As the first line of host defense, activated neutrophils can release NETs, which is defined as decondensed chromatin fibers armed with antimicrobial granular cargos to trap and kill pathogens (Borregaard 2010; Amulic *et al.* 2012). The process of NET release has been defined as "NETosis," an active form of cell death distinct from apoptosis or necrosis (Fuchs *et al.* 2007). Despite their protective role against infection, increasing evidence has suggested that NETs may also promote pathological outcomes in inflammatory and/or thrombotic conditions (Narasaraju *et al.* 2011; Sangaletti *et al.* 2012; von Bruhl *et al.* 2012). However, it is unclear which signaling pathways regulate NETs release in response to inflammatory stimuli. The phorbol ester, phorbol 12-myristate 13-acetate (PMA), is commonly used to induce NETs *in vitro,* whereas variable results have been reported for the induction of NETs by cytokines, chemokines and microorganisms (Remijsen *et al.* 2011). For instance, the bacteria-derived peptide, fMLP, which can potently stimulates reactive oxygen species (ROS) production (Sheppard *et al.* 2005), has been shown by Neeli et al. (Neeli *et al.* 2008) to trigger nuclear chromatin release, while

NET formation via fMLP was not observed by other groups (Urban *et al.* 2009; Remijsen *et al.* 2011).

Recently, mTOR has been shown to regulate lipopolysaccharide (LPS)-induced NETs release by posttranscriptional control of hypoxia-inducible factor 1α protein expression (McInturff *et al.* 2012). However, the authors did not examine the role of mTOR in the autophagy pathway during NETosis. In this study, we test our hypothesis that the mTOR pathway negatively regulates NETs formation via the modulation of autophagic activity in response to fMLP. Our data show that mTOR inhibition with rapamycin accelerated the kinetics of NETs formation upon stimulation with fMLP, which was associated with the rapid formation of autophagosomes. These findings are in contrast to the inhibitory effect of rapamycin in LPS-induced NETosis (McInturff *et al.* 2012). Our data suggest that mTOR activation may prevent neutrophils from undergoing autophagy, thus playing a pivotal role in determining a form of active cell death in activated neutrophils. Moreover, we show that autophagic activity may have a role in histone citrullination, a characteristic histone modification during NETosis, and that NETs formation requires ROS production and functional cytoskeletal machinery, highlighting multiple layers of regulatory mechanisms underlying NETosis.

4.4 Materials and Methods

4.4.1 Reagents

Rapamycin was from LC Labs (Woburn, MA, USA). Anti-microtubule-associated protein light chain 3B (LC3B) antibody (#2775) was from Cell Signaling (Danvers, MA,

USA) and used for autophagosome detection. Anti-Neutrophil elastase (NE) antibody (ab21595) and anti-Histone H3 (citrulline 2 + 8 + 17) antibody (H3Cit; ab5103) were from Abcam (Cambridge, MA, USA) and used as NETs markers. The cell-permeable DNA dye, Hoechst 33342, the cell-impermeable DNA dye, Sytox green, and the intracellular ROS sensor, H_2DCFDA were from Invitrogen (Grand Island, NY, USA).

4.4.2 Live-cell monitoring of NETosis

Purified human neutrophils (2×10^6 /ml) were incubated on fibronectin-coated μ-slide 8 well chamber (ibidi, Verona, WI, USA) at 37°C for 30 min, followed by incubation with indicated inhibitors or vehicle (DMSO) in PMN buffer containing Hoechst 33342 (10 μg/ml) and Sytox green (1 μM). For the detection of intracellular ROS production, adherent neutrophils were loaded with H_2DCFDA (20 μM) for 30 min. Cell medium was replaced with PMN buffer containing inhibitor or vehicle (DMSO) as indicated, and cells were further incubated for 30 min, followed by stimulation with fMLP (1 μM) or PMA (10 nM). Upon the addition of agonists, fluorescent signals were detected using a Zeiss Axiovert fluorescent microscope at various timepoints, and neutrophil morphology was monitored using a DIC microscope. To quantify the kinetics of NETs formation, the number of sytox-positive cells was counted from at least 100 cells per timepoint for each treatment using ImageJ software.

4.4.3 Immunofluorescence microscopy

For NETs detection, primary antibodies were used as follows: anti-NE (1:100), or anti-H3Cit (1:50). For autophagosome detection, fixed cells were permeabilized with

methanol for 3 min, followed by incubation with blocking buffer and anti-LC3B (1:200) antibody. For the quantification of LC3B puncta, the fluorescent signal in all images was adjusted to a fixed threshold, and LC3B-positive particles in the fields of view were counted using a particle analysis function in ImageJ.

4.4.4 Analysis of data

Data are shown as means ± SEM. Statistical analysis was performed using one-way ANOVA or paired Student's t test. Probability values of $P < 0.05$ were selected to be statistically significant.

4.5 Results

4.5.1 mTOR activity regulates the rate of NETosis.

Neutrophils can respond to various stimuli including cytokines and microbial components such as fMLP to elicit anti-bacterial functions (Borregaard 2010; Amulic *et al.* 2012). In neutrophils, fMLP is known to bind the specific G protein-coupled receptors (GPCRs), namely formyl-peptide receptors (FPRs), and activate downstream signaling cascades including the mTOR pathway. The FPR-mTOR signaling axis has been shown to play an important role in neutrophil chemotaxis (Liu *et al.* 2010); however, it is unknown whether mTOR regulates NETs release induced by fMLP. In this study, we first tested whether mTOR activity plays a role in driving NETosis by using the specific pharmacological inhibitor for mTOR, rapamycin and WYE-354. To determine the kinetics of NETs release, extracellular release of DNA from stimulated neutrophils was monitored using the cell-impermeable DNA dye, sytox green, along with the cell-

permeable DNA dye, Hoechst 33342, by direct live-cell imaging. In the presence of fMLP, the percentage of sytox-positive cells remained at minimal levels (Figure 4.1A). Treatment of neutrophils with rapamycin or WYE-354 prior to the addition of fMLP resulted in a significant increase in the sytox-positive population as compared to treatment with vehicle or fMLP alone (Figure 4.1A and 4.1B). In parallel, the protein kinase C (PKC) activator, PMA, potently induced extracellular DNA release in a time-dependent manner (Figure 4.1A). In the absence of agonists, only a minimal increase in the percentage of sytox-positive cells was observed, even after 180 min (8.1 ± 3.8% after 180 min).

To next determine whether extracellular DNA release is caused by NETosis as opposed to apoptotic cell death, we examined the localization of neutrophil elastase (NE), a well-known NETs protein, in neutrophils under basal and mTOR-inhibited conditions. As shown in Figure 4.1C, neutrophils treated with WYE-354 or rapamycin displayed extracellular localization of NE associated with DNA in response to fMLP, suggesting NETs formation. Taken together, these results suggest that mTOR activity negatively regulates the early stage of NETosis and controls the kinetics of NET formation.

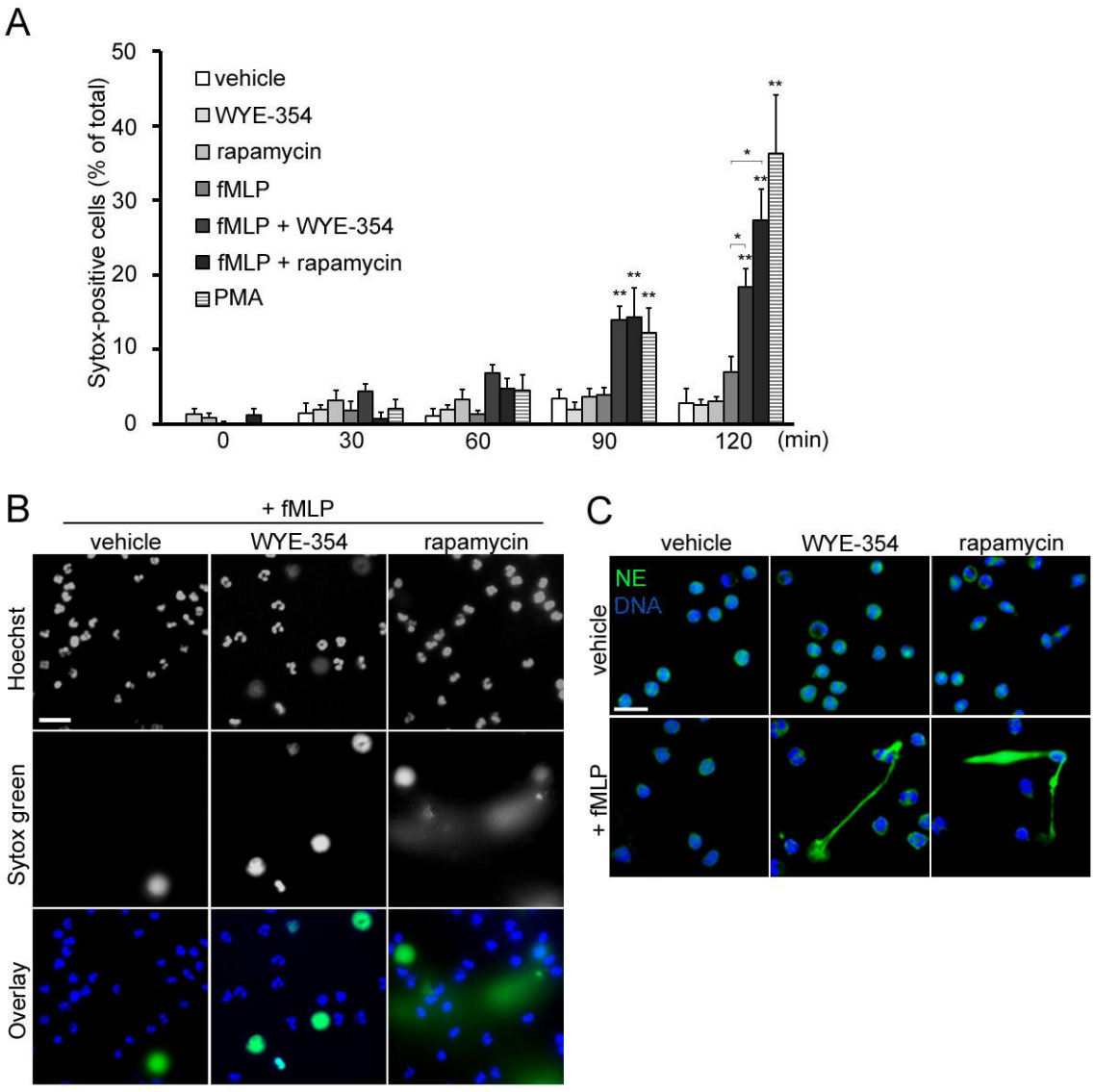

Figure 4.1 mTOR inhibition facilitates the rate of NETs release.

(A) Human neutrophils were treated with vehicle (0.1% DMSO), WYE-354 (30 μM) or rapamycin (100 nM) for 30 min, followed by stimulation with vehicle (HBSS), fMLP (1 μM) or PMA (10 nM). Time-lapse images of intracellular DNA (Hoechst 33342) and extracellular DNA (sytox green) were obtained at every 30 min using a fluorescence microscope. Results were obtained from three independent experiments and represented as the mean percentage of Sytox-positive cells ± SEM. * *P< 0.05*, fMLP-stimulated neutrophils pretreated with vehicle vs. rapamycin or WYE-354; ** *P< 0.05* compared to unstimulated, vehicle-treated neutrophils. (B) Representative images of neutrophils stimulated with fMLP in the presence of vehicle, WYE-354 or rapamycin at 2 h timepoint. (C) Neutrophils treated with vehicle (0.1% DMSO), WYE-354 (30 μM) or rapamycin (100 nM) were incubated with vehicle (HBSS) or fMLP (1 μM), fixed and stained for neutrophil elastase (NE; green) and DNA (Hoechst 33342; blue). Images are representative of at least 3 independent experiments. Scale bar = 20 μm.

4.5.2 mTOR inhibition promotes autophagosome formation

mTOR kinase negatively regulates the translocation of autophagy machinery proteins, such as the ULK complex, to autophagic structures to inhibit the initiation of autophagy (Mizushima 2010; Levine *et al.* 2011). Autophagy has been shown to play a critical role in NETs formation, as inhibition of autophagy by PI3K inhibitors resulted in neutrophil apoptosis (Remijsen *et al.* 2011). Massive cell vacuolization has been described during the early stage of NETosis (Fuchs *et al.* 2007; Remijsen *et al.* 2011), likely due to active autophagic activity. The facilitation of NETs formation by mTOR inhibition led us to question what role the mTOR pathway plays in regulating neutrophil autophagy in response to fMLP. Neutrophils were treated with the PI3K inhibitor, wortmannin, in the presence of rapamycin and stimulated with fMLP in order to assess whether autophagic activity was required for the acceleration of NETosis. Here, NETs were detected as co-localized immunofluorescence of DNA and citrullinated histone H3 (H3Cit), which is known to be generated by peptidylarginine deiminase 4 (PAD4) during NETs formation. As shown in Figure 4.2A, in response to fMLP, a portion of rapamycin-treated neutrophils displayed an increased formation of DNA structures associated with H3Cit, as compared to vehicle-treated cells. In contrast, wortmannin treatment abrogated extracellular DNA release as well as histone citrullination (Figure 4.2A). The inhibitory effect of wortmannin on NETosis was verified in PMA-stimulated neutrophils, in which histone citrullination was absent for cells that were pretreated with wortmannin (Figure 4.2A).

Next, to examine the effect of rapamycin on autophagic activity, neutrophils were stimulated, fixed and stained for microtuble-associated light chain 3B (LC3B) protein. LC3B can be used as a marker of autophagosomes, as it undergoes post-translational modification and associates with autophagic structures upon the initiation of autophagy (Mitroulis *et al.* 2010; Levine *et al.* 2011). Our results show that while LC3B distributed uniformly in the cytosol of neutrophils before the addition of agonists, the immunofluorescence of LC3B was associated with punctate structures after stimulation with fMLP or PMA, suggesting autophagosome formation (Figure 4.2B). In the presence of rapamycin, LC3B accumulation was further increased at 30 min post-addition of fMLP (Figure 4.2B). The formation of LC3B-associated puncta was inhibited in the presence of wortmannin (Figure 4.2B). Overall, these data imply that rapamycin facilitates NETs release via the control of autophagy influx in response to fMLP, and that the mTOR pathway negatively regulates NETosis downstream of FPR signaling.

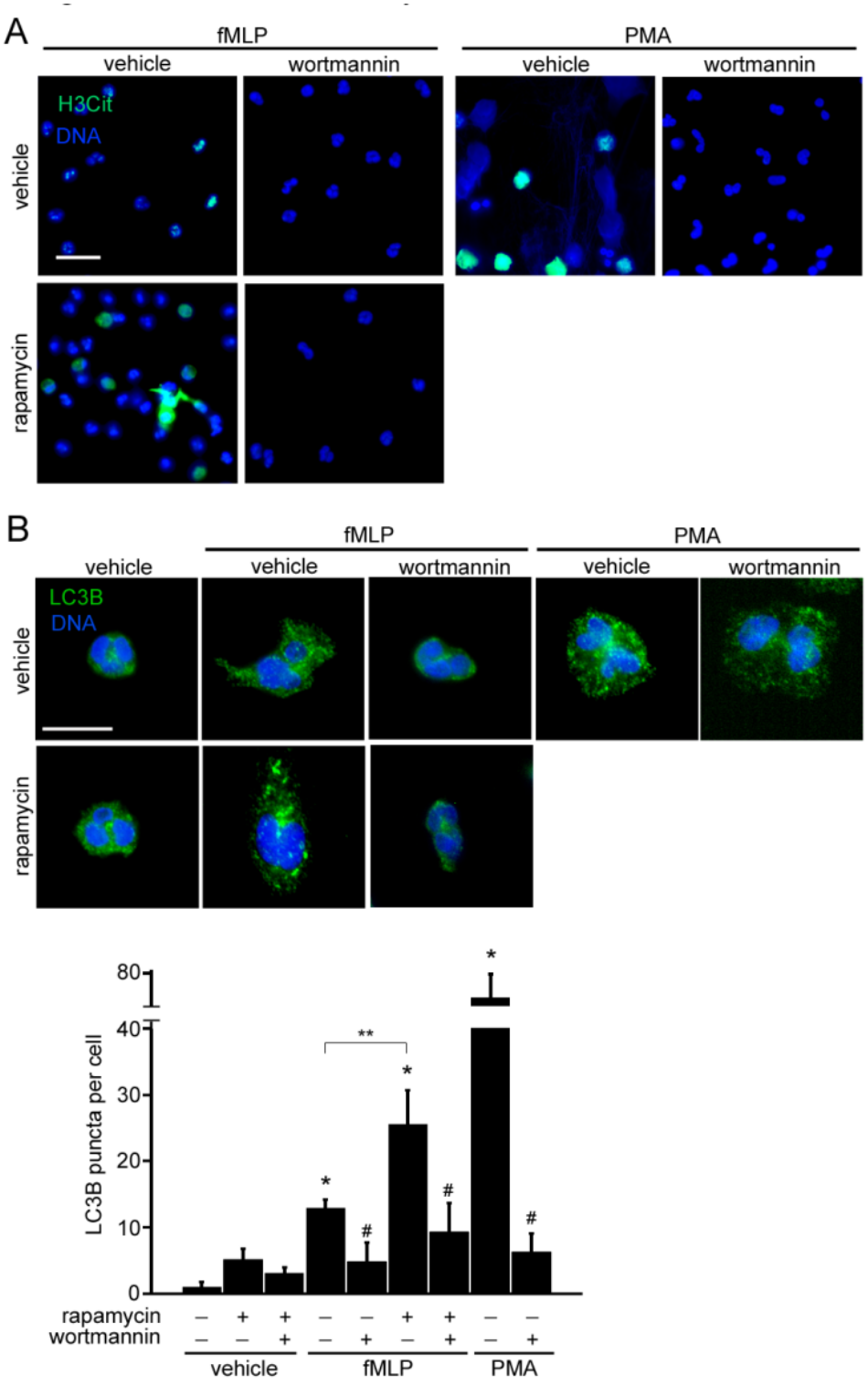

Figure 4.2 Increased autophagic activity by mTOR inhibition mediates NETs release.

(A) Human neutrophils were treated with vehicle (0.1% DMSO) or rapamycin (100 nM) in the presence or absence of the PI3K/autophagy inhibitor, wortmannin (100 nM) for 30 min, followed by stimulation with fMLP (1 μM) or PMA (10 nM) for 2 h. Cells were then stained for

citrullinated histone H3 (histone H3 citrulline 2 + 8 + 17; H3Cit, green) and DNA (blue). (B) Cells were stimulated with fMLP (1 μM) or PMA (10 nM) for 30 min in the presence of vehicle or rapamycin (100 nM) and/or wortmannin (100 nM), and stained for LC3B (green) and DNA (blue). LC3B-positive puncta per cell were quantified using ImageJ. Results are shown as the mean LC3B puncta count ± SEM. * *P<0.05* compared to unstimulated neutrophils; ***P<0.05*, vehicle vs. rapamycin for fMLP-stimulated neutrophils; *#P<0.05*, vehicle vs. wortmannin for fMLP- or PMA-stimulated neutrophils. Results were obtained from 3 independent experiments. Scale bar = (A) 20 μm; (B) 10 μm.

4.5.3 Cytoskeletal machinery is required for NETs formation

Microtubules, actin and myosin play an essential role in neutrophil cytoskeletal reorganization. In addition, these cytoskeletal components contribute to the process of autophagosome formation and lysosomal fusion during autophagy in mammalian cells (Monastyrska *et al.* 2009). To study the role of cytoskeletal reorganization in regulating the autophagic flux downstream of FPR-mTOR signaling pathway, neutrophils were incubated with rapamycin in combination with inhibitors of actin polymerization (cytochalasin D), microtubule polymerization (nocodazole) or myosin II activity (blebbistatin). While rapamycin increased the extent of LC3B-associated puncta in fMLP-stimulated neutrophils, treatment with cytochalasin D, nocodazole or blebbistatin attenuated LC3B accumulation (Figure 4.3A and 4.3B). We next examined whether cytoskeletal machinery was coupled with mTOR-mediated regulation of NETs release. The enhanced formation of DNA meshwork by rapamycin was observed after neutrophil incubation with fMLP for 2 h, as compared to cells treated with fMLP alone (Figure 4.3C). The presence of cytochalasin D, nocodazole or blebbistatin abrogated extracellular DNA release in neutrophils activated by fMLP (Figure 4.3C). Taken together, these results suggest that cytoskeletal machinery is required for rapamycin-enhanced NETs formation, mediated via its regulation of autophagy.

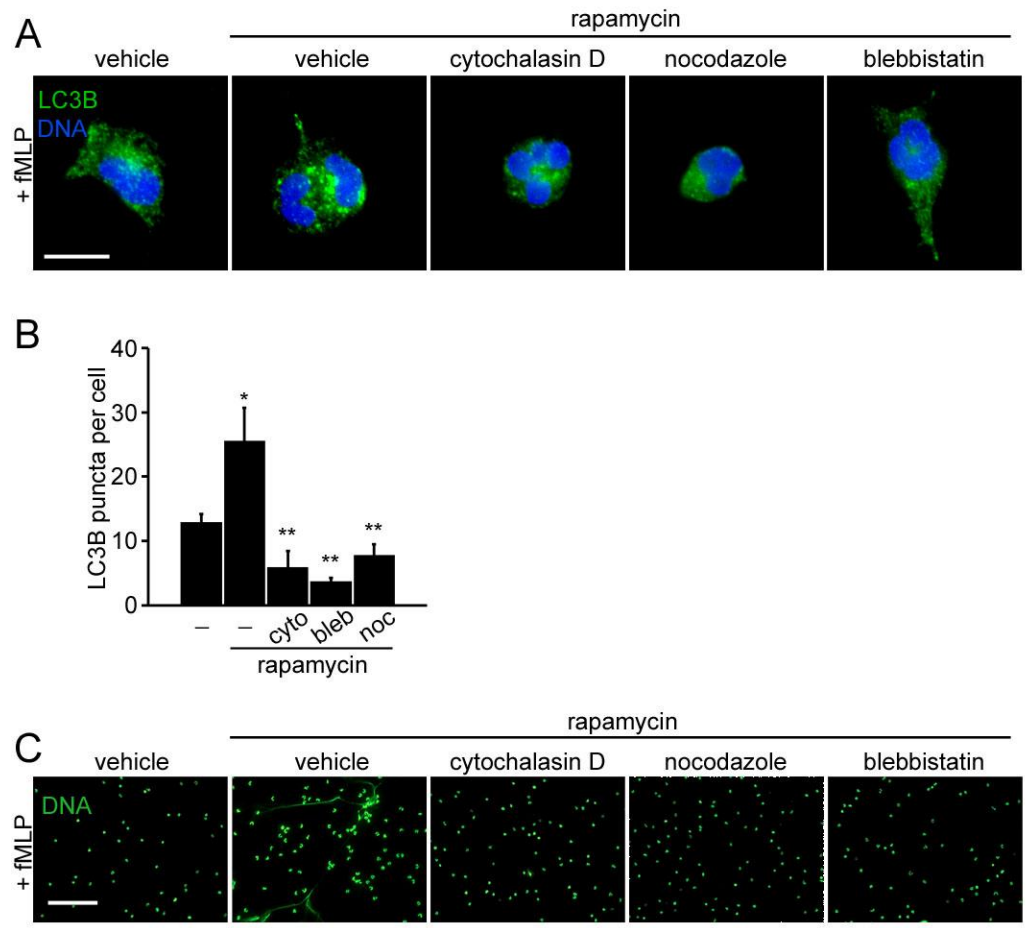

Figure 4.3 Cytoskeletal dynamics are required for autophagy and NETosis.

Human neutrophils were treated with vehicle (0.1% DMSO) or rapamycin (100 nM) in the presence of cytochalasin D (cyto; 10 μM), nocodazole (noc; 10 μM) or blebbistatin (bleb; 100 μM) for 30 min. Cells were stimulated with fMLP (1 μM) for (A) 30 min or (C) 2 h. Fixed cells were stained for (A) LC3B (green) and DNA (Hoechst 33342; blue) or (C) DNA (sytox green; green). (B) LC3B-positive puncta per cell were quantified using ImageJ. Results are shown as the mean LC3B puncta count ± SEM. * *P<0.05* compared to vehicle; ***P<0.05* compared to rapamycin-treated neutrophils. Data shown is representative from 3 independent experiments. Scale bar = (A) 10 μm; (B) 100 μm.

4.5.4 Establishment of the link between the mTOR-axis and ROS production during NETs formation

fMLP, as well as PMA, are also known as potent inducers of respiratory burst, by which neutrophils rapidly produce a large amount of ROS via NADPH oxidase and release ROS to phagosomes or the extracellular environment (Sheppard *et al.* 2005). ROS production

shapes a critical component of NETosis, as neutrophils isolated from chronic granulomatous disease (CGD) patients, who have dysfunctional NADPH oxidase, are unable to form NETs in response to infectious signals (Fuchs *et al.* 2007; Metzler *et al.* 2011; Remijsen *et al.* 2011). In addition, extracellularly added hydrogen peroxide (H_2O_2) is sufficient to trigger NETs (Fuchs *et al.* 2007; Neeli *et al.* 2009). We speculated that altered levels of ROS production are associated with rapid NETosis through mTOR inhibition. Neutrophils were loaded with the ROS sensor, H_2DCFDA, and intracellular ROS production was monitored using live-cell imaging. Intracellular ROS was increased in neutrophils activated by fMLP or PMA as compared to baseline (Figure 4.4A). Rapamycin treatment did not alter basal ROS levels, or fMLP-induced ROS production (Figure 4.4A). We next assessed whether NADPH oxidase activity or extracellular ROS was required for mTOR-mediated promotion of NETosis. While neutrophil treated with rapamycin and fMLP elicited H3Cit-positive NETs structure, the addition of diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase, blocked NETs release and decreased histone citrullination to basal levels (Figure 4.4B). Catalase, which catalyzes the decomposition of H_2O_2, did not abrogate histone citrullination (Figure 4.4B). Moreover, treatment of neutrophils with superoxidase dismutase (SOD), which mediates the dismutation of superoxide anion and produces H_2O_2, promoted H3Cit-associated NETs release (Figure 4.4B). Overall, these results demonstrate an indispensable role of ROS production via NADPH oxidase, likely regulated independently of the mTOR pathway, for NETosis.

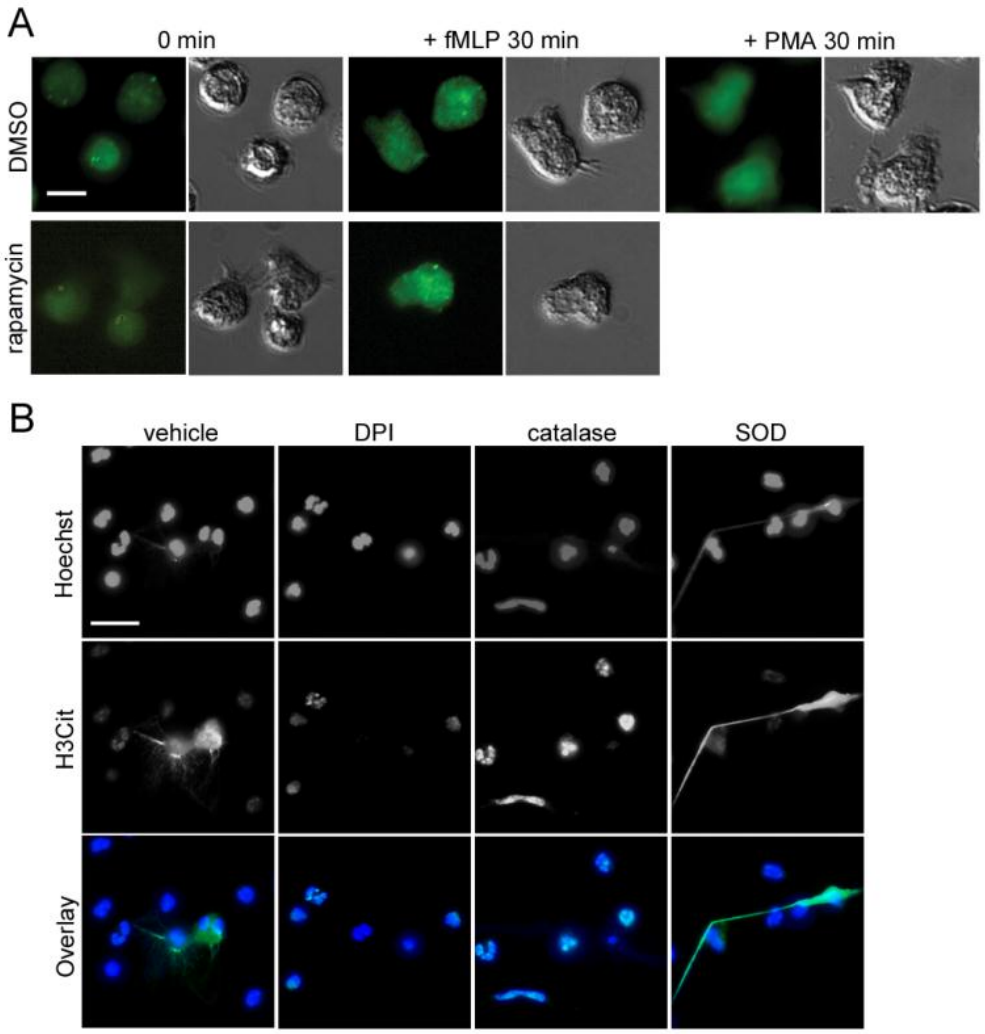

Figure 4.4 mTOR inhibiton does not affect ROS production.

(A) Human neutrophils were loaded with the ROS sensor, H_2DCFDA (20 μM), pretreated with vehicle (0.1% DMSO) or rapamycin (100 nM) for 30 min, and stimulated with (A) fMLP (1 μM) or PMA (10 nM). Representative H_2DCFDA fluorescence images (green) and DIC images are shown at 0 and 30 min after stimulation, obtained from 3 independent experiments. (B) Neutrophils were incubated with rapamycin (100 nM) in the presence of vehicle or the NADPH inhibitor, DPI (10 μM), the H_2O_2 scavenger, catalase (100 U/ml), or the O_2^- scavenger, SOD (80 U/ml), prior to stimulation with fMLP (1 μM) for 2 h. Cells were fixed and stained for citrullinated histone H3 (histone H3 citrulline 2 + 8 + 17; green) and DNA (blue). Scale bar = (A) 10 μm; (B) 20 μm.

4.6 Discussion

In this study, we demonstrate that the mTOR pathway contributes to the regulation of NETs formation via autophagy downstream of FPR signaling in human neutrophils. Our current model of mTOR-mediated NETs regulation is shown in Figure 4.5. As a distinct form of active cell death, NETs formation requires both autophagic activity and ROS production by NADPH oxidase; in fact, the lack of either axis leads to neutrophil apoptosis, followed by secondary necrosis (Remijsen *et al.* 2011). The well-known NETs inducer, PMA, can potently activate PKC, leading to robust ROS production via NADPH oxidase (Sheppard *et al.* 2005), while activating autophagy (Mitroulis *et al.* 2010; Remijsen *et al.* 2011). fMLP binding to FPRs can activate not only NADPH oxidase (Sheppard *et al.* 2005) but also the mTOR pathway (Liu *et al.* 2010), which may inhibit the autophagy pathway. The present study demonstrates that the blockade of mTOR activity by pharmacological inhibitors increases autophagic activity and drives neutrophils towards NETosis. Interdependently of autophagy, the activity of NADPH oxidase and cytoskeletal machinery plays key roles in regulating NETs release.

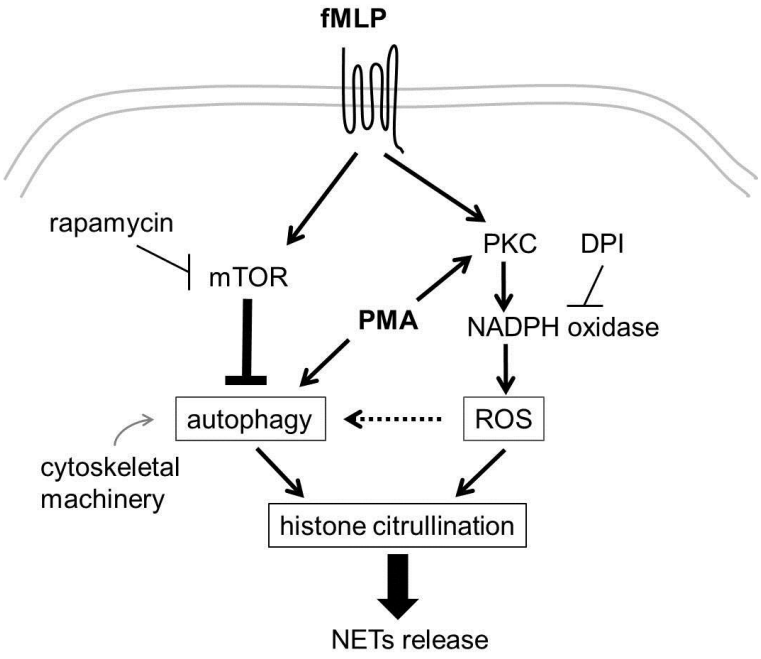

Figure 4.5 Link between mTOR-mediated autophagy regulation and NETs formation in neutrophils.

The present study demonstrated a key role of mTOR in NETs release. fMLP binds to the specific GPCR and potently activates NADPH oxidase, leading to ROS production; in parallel, mTOR activation downstream of fMLP signaling prevents neutrophils from undergoing autophagy. The abrogation of mTOR activity by rapamycin drives autophagy and consequently induces histone citrullination and NETs release in response to fMLP. Cytoskeletal components such as actin, myosin and microtubules are required for autophagy and NETs formation.

A critical role for the mTOR pathway has been shown in the early stage of autophagy by inhibiting the docking of autophagy-related proteins to autophagic structures (Mehrpour *et al.* 2010; Levine *et al.* 2011). In neutrophils, the mTOR pathway is activated in response to various inflammatory stimuli including fMLP, whereas mTOR activation induced by fMLP contributes to cytoskeletal reorganization during chemotaxis via the control of cAMP production and rear contraction (Liu *et al.* 2010). Our results show that mTOR inhibition by rapamycin accelerated NETosis, which paralleled with increased autophagy influx. We propose that this mTOR-dependent regulatory mechanism may explain the inconsistent effects of fMLP on NETs release observed in previous studies

(Neeli *et al.* 2008; Urban *et al.* 2009; Remijsen *et al.* 2011). Contrary to our study, Mclnturff et al. (McInturff *et al.* 2012) reported that rapamycin inhibited NETs release through translational control of hypoxia-inducible factor 1α. It should be noted that the authors of that study induced NETs using LPS, which is a potent inducer of neutrophil autophagy via Toll-like receptor 4 (Huang *et al.* 2009; Mitroulis *et al.* 2010) but is unable to trigger NADPH activity (Sheppard *et al.* 2005). It is not clear whether mTOR activity plays a role in the process of NETosis other than via autophagy regulation. At least in the context of FPR signaling, we show that mTOR activity limits the rate of NETosis via its negative effect on autophagy, while rapamycin treatment does not alter fMLP-induced ROS production. In physiological settings, neutrophils are likely to be exposed to multiple inflammatory signals which would cooperatively activate various cellular functions. Therefore, under certain circumstances, the combination of signal inputs from specific surface receptors including FPR may preferentially drive neutrophils toward an active form of death, NETosis, as opposed to the prolonged survival that allows neutrophils to elicit effective surveillance and clearance.

Histone hypercitrullination by PAD4 has been shown to play a critical role in unfolding chromatin structures downstream of ROS production (Neeli *et al.* 2008; Neeli *et al.* 2009; Wang *et al.* 2009; Rohrbach *et al.* 2012). A recent study has shown that the treatment of a human osteosarcoma cell line with a specific PAD4 inhibitor attenuated cancer cell growth via the downregulation of the mTOR signaling axis, resulting in an increase in autophagy flux (Wang *et al.* 2012). In our study, inhibition of autophagy by wortmannin abrogated histone citrullination and NETs release induced by co-treatment with

rapamycin and fMLP, suggesting an autophagy-dependent mechanism for histone citrullination. It is still unclear whether mTOR can serve to directly regulate PAD4 activity during NETosis. It is possible that the autophagy axis contributes to histone citrullination and subsequent chromatin decondensation by facilitating granule disintegration and PAD4 mobilization in neutrophils during the release of NETs. Notably, actin and microtubule polymerization have shown to be required for histone citrullination via PAD4 during NETs formation (Neeli *et al.* 2009). In this study, we show that inhibition of actin, myosin and microtubule dynamics attenuated fMLP-driven NETs formation in the presence of rapamycin, which was associated with impaired autophagosome formation. Accordingly, the failure of neutrophils to undergo NETosis in the presence of cytoskeletal inhibitors may be due to disrupted autophagic processes interdependently of histone citrullination. We propose that the accelerated NETs release in the absence of mTOR activity may depend on cytoskeletal machinery at multiple stages of NETosis.

Pathological roles of NETs have been reported in the contexts of autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), where many autoantibodies are developed against citrullinated self-antigens (e.g. histones) and/or neutrophil proteins including NE, myeloperoxidase, and proteinase-3 (Knight *et al.* 2012; Sangaletti *et al.* 2012). Impairment of NETs degradation has found in SLE patients, suggesting that NETs contributes to the development of autoantibodies by providing autoantigens and damage-associated molecular patterns (Hakkim *et al.* 2010). While autophagy is known to facilitate antigen presentation in macrophages and B-cells during

SLE and RA (Jones *et al.* 2013), our study indicates that dysregulated autophagic activity in neutrophils may promote the exposure of citrullinated histones in these autoimmune diseases.

In summary, the present study reveals a complex set of regulatory mechanisms, including the autophagy pathway, underlying NETs formation in activated human neutrophils. As the ability of neutrophils to combat against infection is not limited to NETs release (i.e. degranulation, phagocytosis), future studies will be focused on elucidating the missing links explaining how neutrophils have evolved a strategy for pathogen clearance in response to inflammatory stimuli.

Chapter 5: Activated Factor XI Inhibits Chemotaxis of Neutrophils

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5.1 Abstract

Neutrophils are the most abundant leukocytes in the circulation and play an important role in host defense. Neutrophil recruitment and inflammatory responses at sites of infection are critical components in innate immunity. Although inflammation and coagulation are known to have bidirectional relationships, little is known about the interaction between neutrophils and coagulation factors. Coagulation factor XI (FXI) participates in the intrinsic coagulation pathway upon its activation, contributing to hemostasis and thrombosis. We have previously shown that FXI-deficient mice have an increased survival and less leukocyte accumulation into the peritoneum in severe polymicrobial peritonitis. This result suggests a role for FXI in leukocyte trafficking and/or function. In this study, we characterized the functional consequences of activated FXI (FXIa) binding to neutrophils. FXIa reduced neutrophil chemotaxis triggered by the chemokine, interleukin-8 (IL-8) or the bacterial-derived peptide, formyl-Met-Leu-Phe (fMLP), perhaps due to the loss of directed migration. In summary, our data suggest that FXIa modulates the inflammatory response of neutrophils by altering migration. These studies highlight the interplay between inflammation and coagulation, and suggest that FXIa may play a role in innate immunity.

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5.2 Introduction

Studies in Chapter 3 and 4 were conducted to elucidate the signaling crosstalk and cellular events that regulate neutrophil functions in response to the end-target chemoattractant, fMLP. The activation of the coagulation system and inflammation is interdependently regulated as a function of host defense, where excessive neutrophil tissue infiltration can contribute to the pathogenesis of clinical conditions such as sepsis. In this chapter, studies characterize the role of the coagulation factor XIa (FXIa), a serine protease that plays a role in the amplification phase of coagulation, in the regulation of neutrophil activation and chemotaxis.

5.3 Background

Neutrophils play a central role in innate immunity, and are responsible for mediating the inflammatory response to bacterial infection and tissue injury (Borregaard 2010). Neutrophil recruitment and transmigration across the endothelial cell lining of the vasculature is orchestrated by the secretion of chemoattractants and cytokines from infected, inflamed or injured tissues. The anti-microbial activity of neutrophils is characterized by phagocytosis, degranulation of cytolytic enzymes and production of superoxide anion. The anti-microbial function of neutrophils is critical to host defense

against bacterial infections, however, dysregulation of neutrophil influx can result in severe collateral tissue damage (Reddy and Standiford 2010).

Enzymes of the blood coagulation cascade are known to be activated at sites of inflammation (Levi and van der Poll 2005), where they are thought to play a role in regulating the trafficking and function of neutrophils. For instance, mice deficient in coagulation factor XI (FXI) have higher survival rates after induction of severe polymicrobial peritonitis and reduced leukocyte accumulation at the site of infection (Tucker *et al.* 2008). However, it is not known whether coagulation factors, such as FXI, play a direct role in the regulation of neutrophil defense mechanisms.

FXI is a 160 kDa homodimer that plays a key role in the early phase of the intrinsic blood coagulation cascade (Emsley *et al.* 2010). In the intrinsic phase, FXI is activated to FXIa either by activated factor XII (FXIIa), thrombin or by autocatalytic activation. FXIa functions as a serine protease to cleave and activate factor IX, leading to sustained thrombin generation. FXI circulates in the plasma as a complex with high molecular weight kininogen (HK), a feature shared in common with prekallikrein (PK), the monomeric homolog of FXI. Neutrophils have been reported to bind FXI directly (Henderson *et al.* 1994); however, the functional consequences of FXI-neutrophil interaction have not been described. In this study, our data suggest that FXIa modulates the inflammatory response of neutrophils by altering migration. These studies highlight the interplay between inflammation and coagulation, and suggest that FXIa may play a role in innate immunity.

5.4 Materials and Methods

5.4.1 Reagents

Plasma-derived FXIa and factor XII (FXII) were purchased from Hematologic Technologies Inc (Essex Junction, VT, USA). HK and PK were purchased from Enzyme Research Laboratories Inc (South Bend, IN, USA).

5.4.2 Static adhesion assays

Non-tissue culture treated 24-well plates were incubated with proteins for 1 h at room temperature. Surfaces were then blocked with BSA for 1 h and washed with PBS. Purified human neutrophils (1×10^6 /ml) were incubated on protein-coated surfaces at 37°C for 30 min. After washing with PBS and fixation with 4 % paraformaldehyde, adherent cells were imaged using 10× phase microscopy and counted using ImageJ software.

5.4.3 Transwell assays

Both upper and bottom chambers of 24 well Transwell plates (Corning; 3.0 μm pore) were coated with fibronectin (5 μg/ml) for 1 h, washed with PBS and dried overnight. After incubation with indicated reagents, purified neutrophils were placed onto the upper chamber, in the presence of fMLP (10 nM) or IL-8 (100 ng/ml) in the bottom chamber and incubated at 37 °C for 90 min. After incubation, EDTA (final 50 mM) was added to the bottom chamber at 4 °C for 10 min. The supernatants in the bottom chambers were collected and pelleted by centrifugation at 500 g for 10 min. Migratory cells were resuspended and counted using a hemocytometer.

5.4.4 Analysis of data

Data are shown as means ± SEM. Statistical analysis was performed using paired Student's t test. Probability values of $P < 0.05$ were selected to be statistically significant.

5.5 Results

5.5.1 Characterization of FXIa-neutrophil interactions

We first performed static adhesion assays using purified human neutrophils to evaluate the ability of neutrophils to bind FXIa. We found that immobilized FXIa supported binding of 59.7 cells/ mm^2 (Figure 5.1). A similar degree of neutrophil binding was observed on fibronectin or synthetic polypeptide analogues containing the Arg-Gly-Asp (RGD) sequence found in the functional domain of fibronectin. In contrast, only a limited degree of neutrophil adhesion was observed to immobilized PK, which has structural similarity to FXI (Emsley *et al.* 2010), or FXII (Figure 5.1B).

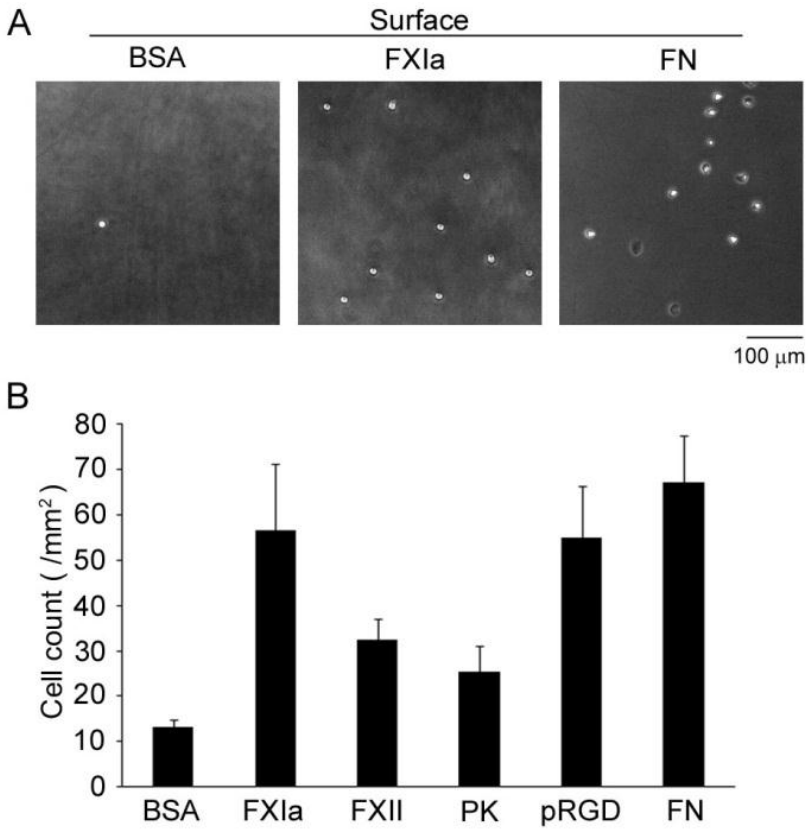

Figure 5.1 Human neutrophil binding to FXIa.

Purified human neutrophils were placed on protein-coated surfaces for 30 min at 37°C and imaged using 10× phase microscopy for quantification. (A) Representative images of phase-bright PMNs on BSA (2% w/v), FXIa (50 μg/ml) or fibronectin (FN; 50 μg/ml)-coated surfaces. (B) Adherent PMNs on immobilized BSA, FXIa, FXII (50 μg/ml), prekallikrein (PK; 50 μg/ml), polyRGD (pRGD; 20 μg/ml) or FN (50 μg/ml) were calculated and are reported as mean cell count/ mm^2 ± SEM. Results were obtained from at least 3 independent experiments.

We next investigated the molecular mechanisms of FXIa binding to neutrophils. As shown in Figure 5.2, the serine protease inhibitor PPACK reduced neutrophil binding to FXIa by over 50%, suggesting that protease activity of FXIa, or alternatively its catalytic domain, plays a role in FXIa-neutrophil binding. Equivalent results were observed whether PPACK was present in solution (31.3 cells/ mm^2) or if FXIa was pretreated with PPACK (21.4 cells/ mm^2). In contrast, chelation of extracellular divalent cations with

EDTA or saturation of surface expressed phosphatidylserine with Annexin V did not affect neutrophil binding to FXIa (Figure 5.2).

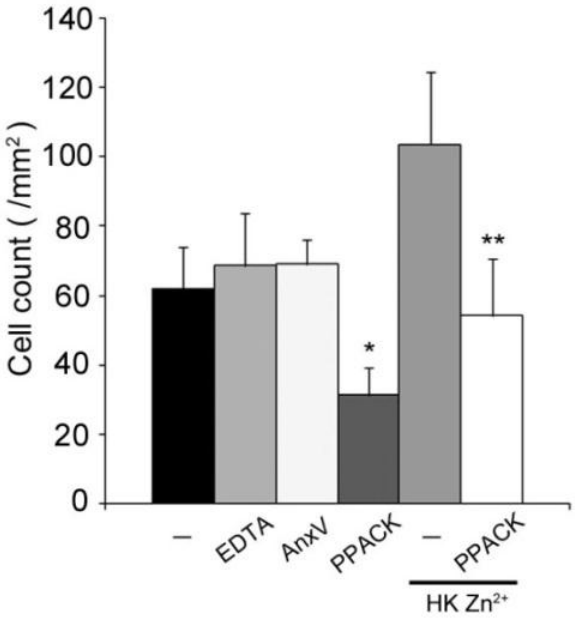

Figure 5.2 Characterization of neutrophil-FXIa interaction.

Purified human neutrophils were incubated over surfaces of FXIa (50 μg/ml) for 30 min at 37°C in the presence of vehicle (PMN buffer; –), EDTA (5 mM), AnnexinV (AnxV; 10 μg/ml), or serine protease inhibitor PPACK (40 μM). In selected experiments, cells were treated with Zn^{2+} (25 μM) and high molecular weight kininogen (HK; 42 nM). Results are reported as mean cell count/ mm^2 ± SEM of 3 independent experiments. $P < 0.05$ compared to adhesion of vehicle-treated cells in the absence (*) or presence (**) of Zn^{2+}/HK.

Zymogen FXI circulates in complex with HK. In the presence of the divalent cation Zn^{2+}, HK has been shown to regulate FXIa binding to blood platelets (Greengard *et al.* 1986; Baird and Walsh 2002; White-Adams *et al.* 2009) and endothelial cells (Baird and Walsh 2002). Along these lines, our data show that the presence of HK/Zn^{2+} enhanced neutrophil binding of FXIa (Figure 5.2). This interaction remained sensitive to the presence of PPACK.

5.5.2 Attenuation of fMLP-induced Ca^{2+} mobilization by FXIa

It is known that neutrophil activation is associated with an increase in cytosolic Ca^{2+}, resulting from the mobilization of intracellular Ca^{2+} stores. In agreement with previous reports, our data show that the exposure of neutrophils to the bacterial peptide, fMLP, resulted in a rapid spike in intracellular Ca^{2+} (Figure 5.3), followed by a rapid decay in intracellular Ca^{2+} (exponential time constant = 3.29 ± 0.22 min). Experiments were designed to determine whether FXIa regulates fMLP-induced Ca^{2+} mobilization. Our data showed that FXIa substantially inhibited neutrophil intracellular Ca^{2+} mobilization in response to fMLP. FXIa alone did not induce intracellular Ca^{2+} mobilization (Figure 5.3, first arrow). Taken together, our data suggests that FXIa sequesters the release of intracellular Ca^{2+} induced by fMLP.

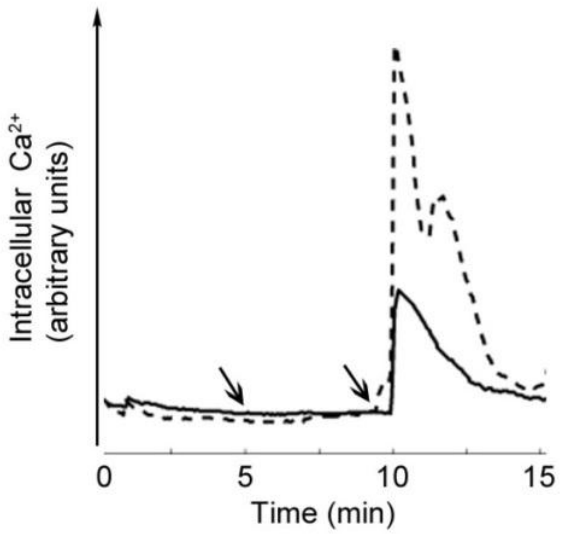

Figure 5.3 FXIa attenuates fMLP-induced Ca^{2+} mobilization.

Human neutrophils were loaded with fluo-4 AM (2 μM) and plated on fibronectin-surface. Intracellular Ca^{2+} spikes were monitored for 15 min. Vehicle (PMN buffer; dashed line) or FXIa (10 μg/ml; black line) was added at 5 min (first arrow) followed by the addition of fMLP (10 nM) at 10 min (second arrow). Data is presented as mean intensity of 5 neutrophils in a field of view from each treatment.

5.5.3 Inhibition of directional migration by FXIa

It is well established that neutrophils migrate to the sites of inflammation in the response to chemoattractants such as fMLP and IL-8. We assessed the effect of FXIa on neutrophil chemotaxis towards fMLP and IL-8 using a Transwell assay. Our data show that FXIa reduced neutrophil migration to fMLP or IL-8 by ~30% (Figure 5.4A and 5.4B, respectively). In agreement with previous studies, we show that tumor necrosis factor α (TNFα) abrogated the chemotactic activity of fMLP (Figure 5.4A). In contrast, PK failed to inhibit fMLP-driven chemotaxis (Figure 5.4).

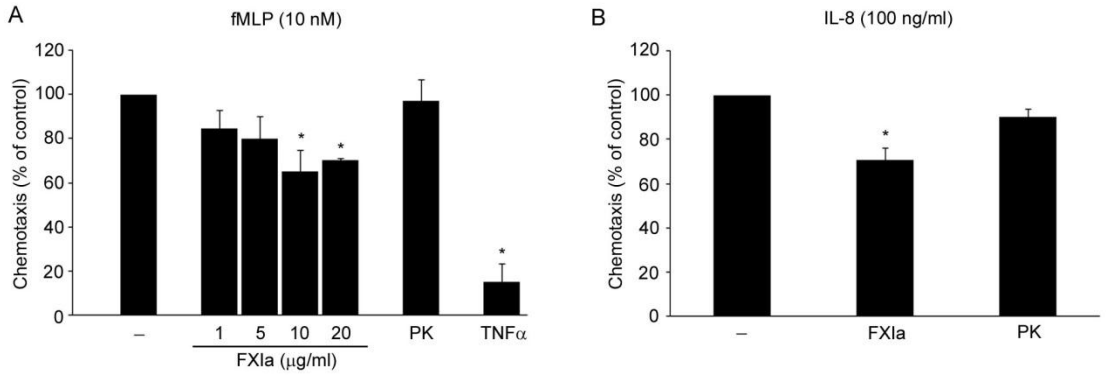

Figure 5.4 Inhibitory effects on fMLP-driven neutrophil chemotaxis.

(A) Human neutrophils pretreated with vehicle (PMN buffer; –), FXIa (1, 5, 10, 20 μg/ml), PK (20 μg/ml) or TNFα (10 ng/ml) were placed on the fibronectin (5 μg/ml)-coated transwell inserts and allowed to migrate towards fMLP (10 nM)-containing bottom chamber for 90 min at 37°C. (B) Neutrophils pretreated with vehicle, FXIa (10 μg/ml) or PK (10 μg/ml) were allowed to migrate towards IL-8 (100 ng/ml)-containing bottom chamber for 90 min at 37°C.The results are presented as mean percentage ± SEM of migratory population for each treatment relative to chemoattractant-driven chemotaxis. * $P < 0.05$ compared to the migration of vehicle-treated cells to the indicated chemoattractants.

Next we directly visualized and tracked migrating neutrophils in real-time using an Insall chamber (Muinonen-Martin *et al.* 2010). The linear gradient of chemoattractant was established by adding fMLP to the outer well and the time-lapse images were taken at the

central area of the wide (1.0 mm) bridge for 30 min. We observed directional migration of vehicle-treated neutrophils toward the chemoattractant gradient of fMLP (Figure 5.5). FXIa significantly diminished neutrophil migration towards the fMLP gradient, resulting in random movement (Figure 5.5). The inhibitory effect of FXIa treatment was further confirmed by Rayleigh's test, vehicle ($p = 3.43E-15$) versus FXIa ($p = 0.89$), indicating that vehicle-treated neutrophils demonstrated a highly significant unimodal deviation (directed migration) from a uniform angular distribution (random migration). The mean resultant vector (arrows in Figure 5.5B) for vehicle-treated neutrophils had a magnitude of 0.916 and pointed in the direction of the fMLP gradient (275°) with narrow 95% confidence bounds (± 9.5°) while the FXIa mean resultant vector was smaller in magnitude 0.062 and pointed away from the gradient (310°) with larger 95% confidence bounds (± 86°).

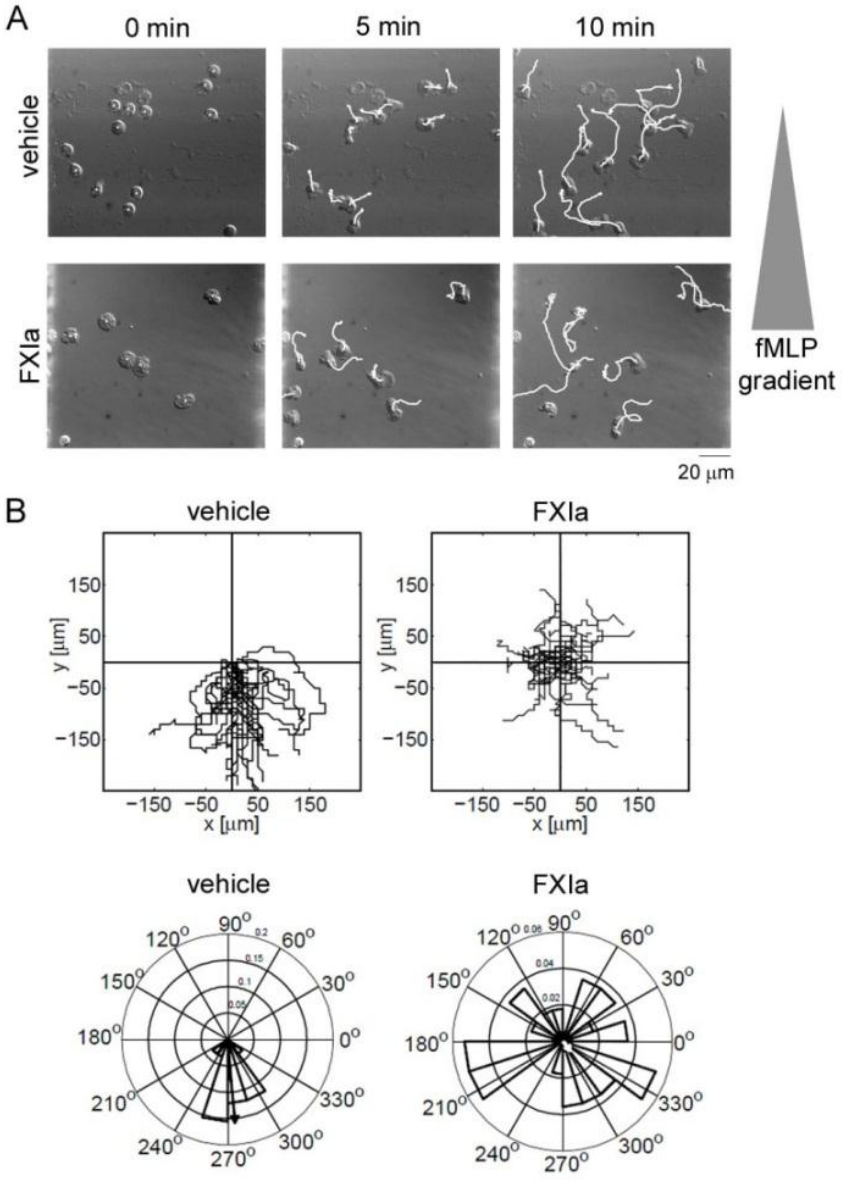

Figure 5.5 FXIa inhibits directional migration.

Human neutrophils on fibronectin-coated coverslips were pretreated with vehicle (PMN buffer) or FXIa (10 μg/ml) and placed on Insall chamber. Chemotaxis was induced by adding fMLP (10 nM) in the outer well of the chamber. Time-lapse images were taken every 10 s for 30 min. (A) Time-lapse images at first 0, 5 and 10 min with cell paths (white lines). (B) The neutrophil migration was analyzed using the ImageJ plugin MtrackJ and MATLAB. Spider plots indicate the paths of individual cells (upper panels) and rose plots indicate the angular histograms associated with the trajectories (lower panels). The mean resultant vectors are shown in rose plots (arrows).

5.6 Discussion

Neutrophils are key mediators of inflammation, and their migration is strictly regulated for both host defense and the prevention of tissue damage. In this study, we showed that coagulation factor XIa binds to human neutrophils and modulates chemotaxis.

Crosstalk between coagulation and inflammation is increasingly thought to be mediated by the contact system, which consists of FXII, FXI, PK and HK (Colman and Schmaier 1997). The activation of FXII leads to the enzymatic generation of FXIa from FXI, as well as the generation of kallikrein from PK. These enzymes in turn are responsible for generation of factor IXa (FIXa), the activated form of coagulation factor IX, and generation of the potent inflammatory and vasodilator peptide, bradykinin (BK), from HK. Local accumulation of BK triggers the classical signs of inflammation, including vasodilation, increased permeability, pain and reduced tissue function. FIXa can activate the common pathway of blood coagulation, leading to the generation of the enzyme, thrombin. Thrombin generation can then lead to platelet activation, fibrin formation, and vaso-occlusive thrombi.

In patients with a clinical suspicion of disseminated intravascular coagulation, markers of contact system activation are elevated (Kaufman *et al.* 1991; Wuillemin *et al.* 1995). In a baboon model of lethal bacteremia, an inhibitory monoclonal antibody against FXII extended animal survival time (Pixley *et al.* 1993). Even though the limited treatment failed to prevent disseminated intravascular coagulation, the data suggested that reducing FXII activity reduced the systemic inflammatory response to sepsis. FXII has been shown

to induce monocyte IL-1 production and secretion (Toossi *et al.* 1992), neutrophil aggregation and degranulation (Wachtfogel *et al.* 1986), and complement activation (Ghebrehiwet *et al.* 1981). Moreover, HK has been shown to drive the secretion of proinflammatory cytokines such as TNFα, IL-6 and IL-8 from human mononuclear cells (Khan *et al.* 2006). Our study provides the first evidence that FXIa modulates neutrophil inflammatory responses. It remains to be determined which receptors and signaling pathway(s) are involved in this interaction. Future studies will be aimed to identify the molecular mechanisms that contribute to the action of FXIa on neutrophils.

Sepsis is characterized by the failure to maintain the necessary balance between excessive and inadequate neutrophil migration to tissues. We have previously shown that FXI-deficient mice have increased survival relative to wild-type mice in a model of severe polymicrobial peritonitis, with FXI-deficient mice exhibiting a reduction in coagulopathy and leukocyte infiltration at the site of infection (Tucker *et al.* 2008). Another study using mice deficient in both plasminogen (Plg) and FXI found an increased leukocyte infiltration in lungs of $Plg^{-/-}$ $FXI^{-/-}$ mice in contrast to $Plg^{-/-}$ $FXI^{+/+}$ mice (Cheng *et al.* 2005). Our *in vitro* experiments in the present study demonstrate the inhibitory effect of FXIa on human neutrophil chemotaxis to the exogenous chemotactic factors, fMLP and IL-8. These findings imply that FXIa may arrest migrating neutrophils to those sites where coagulation is initiated. While inflammation promotes the activation of coagulation, perhaps accumulated FXIa on the clot surface promotes neutrophil retention by reducing their ability to leave the area. FXIa may also promote additional coagulation by limiting neutrophil migration and concentrating neutrophil associated tissue factor

(Giesen *et al.* 1999; Maugeri *et al.* 2006), which is a key driver of pathological coagulation (Todoroki *et al.* 2000). These data implicate a potential mechanism of disease development via the coupling of coagulation and inflammation in sepsis.

Chapter 6: Conclusions and Future Work

6.1 Summary

Studies in Chapter 3-5 characterize the regulatory mechanisms of human neutrophil response to (patho)physiologic triggers, including activation, migration and NETosis.

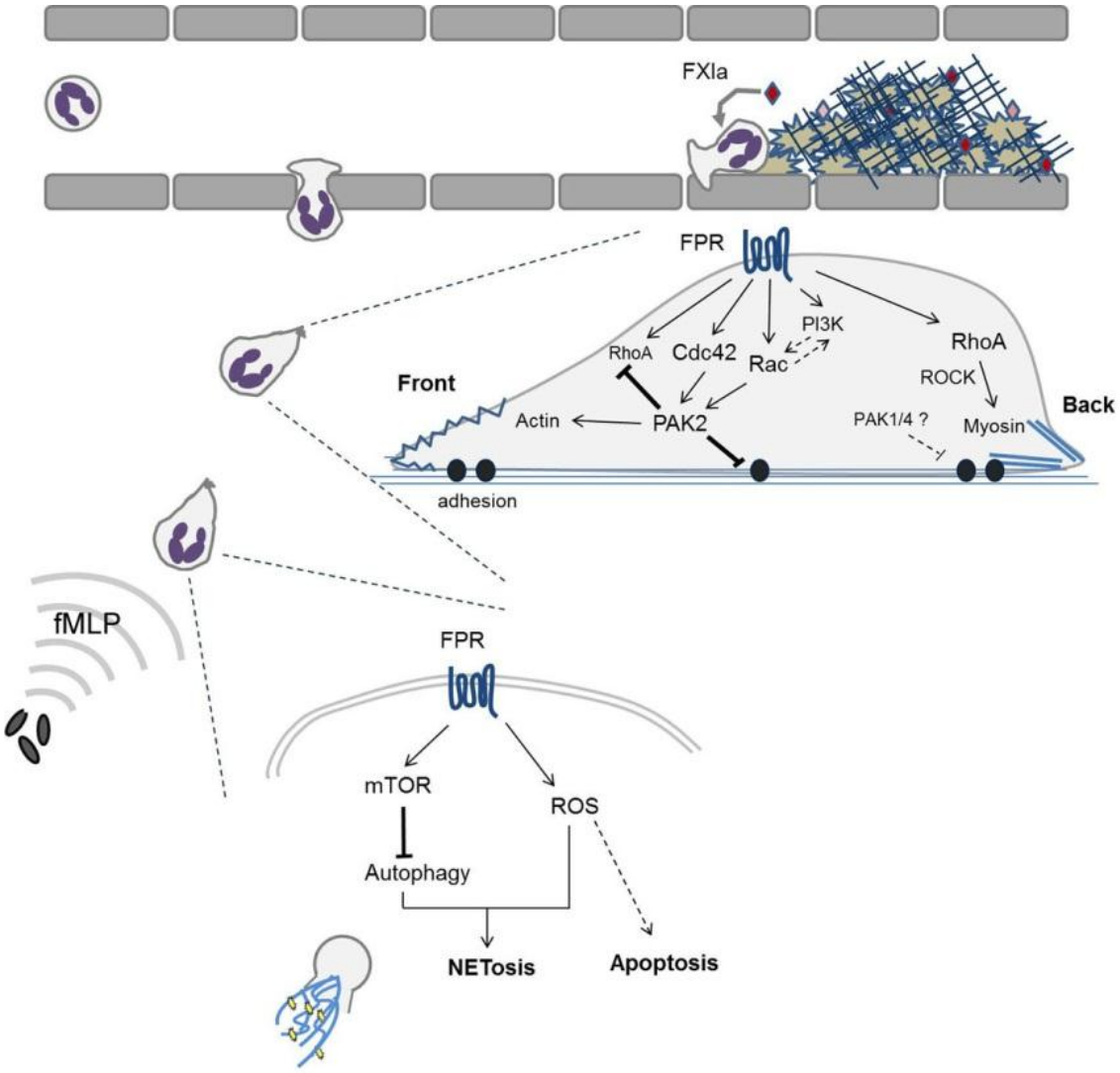

Figure 6.1 Unified model of the regulatory mechanisms of human neutrophil response.

Studies in this thesis identify key mechanisms underlying neutrophil function: p21-activated kinase (PAK), as a key coordinator of cytoskeletal dynamics during cytoskeletal reorganization (Chapter 3); the mammalian target of rapamycin (mTOR) pathway, as a pivotal point of neutrophil fate decision towards NETosis (Chapter 4); and activated coagulation factor XI (FXIa) in the control of neutrophil activation and chemotaxis (Chapter 5). Building on the findings in this thesis, future studies will increase our knowledge of neutrophil-driven pathophysiological processes, which could be translated to novel therapeutic approaches to tackle clinical complications associated with inflammation.

The following section discusses several immediate and long-term research questions that remain to be addressed in order to extend the findings of this thesis.

6.2 Regulation of Neutrophil Functions by the Intrinsic Coagulation Pathways

As shown in Chapter 5, FXIa can bind to neutrophils and inhibit their migratory ability in response to fMLP or IL-8. However, the molecular mechanisms that mediate FXIa-neutrophil interactions remain unclear. Results in Chapter 5 suggest that the protease activity of FXIa is required for neutrophil binding to FXIa. First, it is hypothesized that FXIa downregulates the expression of functional chemoattractant receptors, by enzymatically cleaving receptors and/or inducing receptor internalization. To address this hypothesis, preliminary studies were performed to determine the surface expression of the formyl-peptide receptor, FPR, using flow cytometry. As compared to the basal FPR expression, neutrophil treatment with FXIa did not cause any detectable changes in the

surface FPR level, regardless of whether neutrophils were stimulated with fMLP and IL-8 (Fig. 6.2). Control experiments performed in the presence of PPACK, which blocks the enzymatic function of serine proteases, demonstrate that the surface expression of FPR was not affected by the enzymatic activity of FXIa (Fig. 6.2). These results indicate that the action of FXIa on neutrophils is unlikely to be mediated via the regulation of chemoattractant receptor expression.

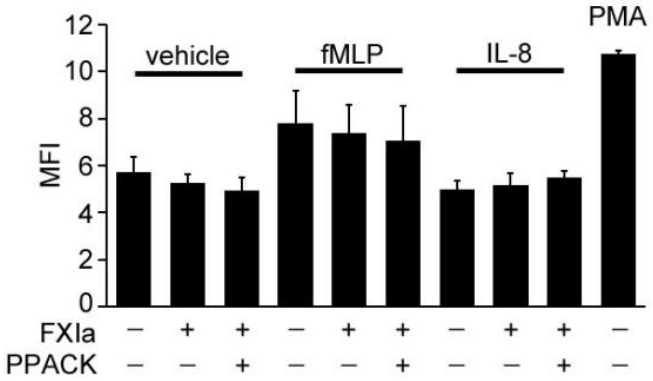

Figure 6.2 Neutrophil expression of formyl-peptide receptor (FPR) was not altered by FXIa.

Neutrophils were treated with vehicle or FXIa (10 μg/ml) for 30 min in the presence or absence of PPACK (40 μM), and stimulated with vehicle, fMLP (10 nM) or IL-8 (100 ng/ml) for 15 min. As a positive control, neutrophils were treated with PMA (100 nM) for 5 min. Cell surface FPRs were labeled using PE mouse anti-human fMLP receptor antibody (BD Pharmigen) and detected using FACS Calibur.

The next hypothesis is that, among potential FXIa substrates, coagulation factor FXII expressed on the neutrophil surface (Henderson *et al.* 1994) could be cleaved and activated by FXIa. As a result, the local formation of FXIIa may serve to attenuate fMLP-triggered responses in neutrophils. Data from preliminary chemotaxis assays designed to address this hypothesis demonstrate that in contrast to the random migration observed for FXIa-treated neutrophils, the addition of PPACK together with FXIa restored normal neutrophil directional migration towards the source of fMLP (Fig. 6.3A). Interestingly,

the selective FXIIa inhibitor, corn trypsin inhibitor (CTI), abrogated the ability of FXIa to impair neutrophil chemotaxis towards an fMLP gradient (Fig. 6.3A). In a purified system, the serine protease inhibitors, aprotinin and PPACK, but not CTI, directly inhibited FXIa enzymatic activity (Fig. 6.3B). These results together indicate that FXIIa activity is involved in the inhibitory effect of FXIa on neutrophil chemotaxis. Conversely, exogenously added α-FXIIa did not affect fMLP-driven directional migration (Fig. 6.3A). Future experiments are required to determine why exogenously added α-FXIIa does not recapitulate the phenotype of FXIIa being formed on the neutrophil surface by FXIa. One of the hypothesized mechanisms is that FXIa generates specific cleavage products derived from zymogen FXII on the neutrophil surface. Along these lines, it has been shown that the activation of the zymogen FXII leads to the formation of two distinct forms of the active enzyme: α-FXII, that consists of a heavy chain and a light chain, and β-FXIIa, that only consists of a light chain containing the FXII catalytic region. Notably, β-FXIIa can activate the inflammatory kallikrein–kinin system, but not coagulation, due to the lack of binding regions for negatively-charged surfaces (Stavrou and Schmaier 2010). It is possible that FXIa acts on the neutrophil surface FXII to form β-FXIIa. Alternatively, it has been suggested that bone marrow-derived FXII may have a distinct role in leukocyte tissue infiltration during wound healing, although the structural and functional differences between bone marrow-derived FXII and plasma FXII is ill-defined (Dr. Schmaier, personal communication). In future studies, the effect of exogenous β-FXIIa will be tested in the chemotaxis assay, and protein fragments generated via the proteolytic cleavage by FXIa will be collected from neutrophil lysates and in a purified system containing FXII, in order to determine the cleavage products by utilizing mass-

spectrometry analysis. These future studies will provide an understanding of the regulatory role of the intrinsic coagulation pathway in inflammation.

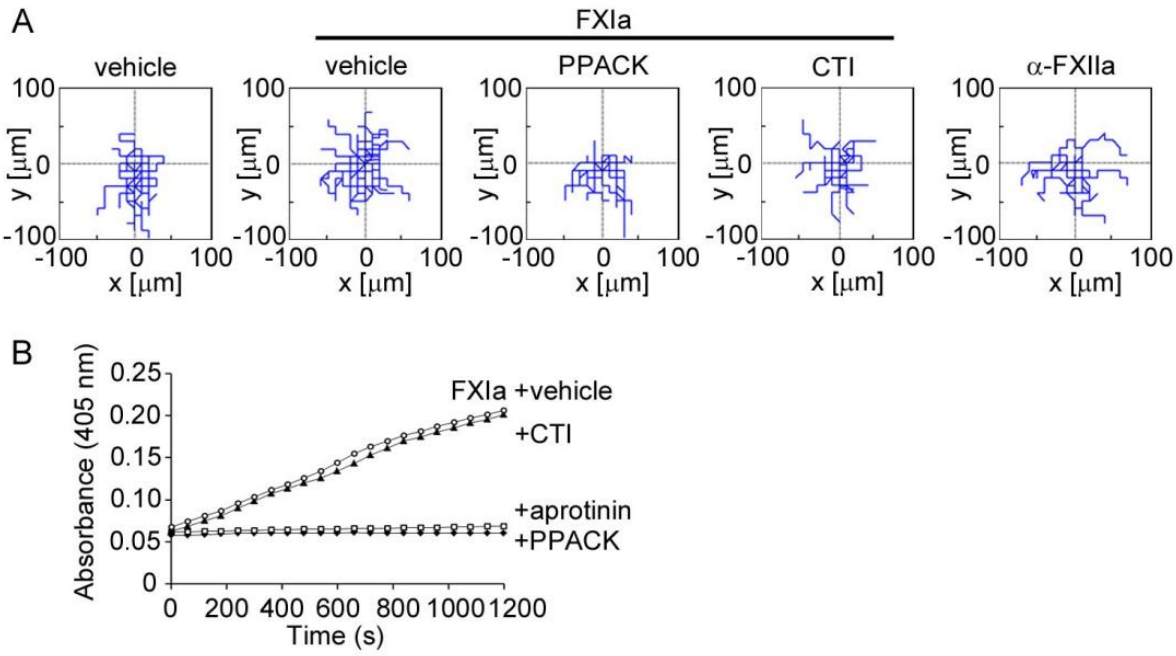

Figure 6.3 The inhibitory effect of FXIa on chemotaxis requires the protease activity of FXIa.

(A) Human neutrophils were treated with vehicle (PMN buffer), FXIa (10 μg/ml) or α-FXIIa (10 μg/ml) for 30 min and chemotaxis was induced by adding fMLP (10 nM) in the outer well of an Insall chamber. In selected experiments, PPACK (40 μM) or CTI (20 μg/ml) was added prior to neutrophil incubation with FXIa. Time-lapse images were obtained every 10 s for 20 min at 40× magnification using DIC microscopy and the migration paths of individual cells were quantified as spider plots using MtrackJ (ImageJ) and MATLAB. (B) Enzymatic activity of FXIa was quantified in a purified system by measuring the hydrolysis rates of the chromogenic substrate S-2366 at 405 nm in the presence of vehicle (○), CTI (20 μg/ml;▲), aprotinin (10 μM;□), or PPACK (40 μM;♦).

6.3 (Patho)physiological Roles of Neutrophils

6.3.1 NETs-mediated prothrombotic activity

NETs provide a structural framework to localize pathogens and stimulatory factors for efficient pathogen clearance. However, recent studies have highlighted a role of NETs beyond a direct host defense function, and reported that NETs can contribute to the

pathology of the vascular system and promote prothrombotic states including deep vein thrombosis. Studies in Chapter 4 describe the signaling pathways involved in the neutrophil fate decision towards NETosis. Future studies will be designed to determine the pathological roles of neutrophils, and more specifically, how the "off-target" effects of NETs can lead to the development of prothrombotic conditions. In preliminary studies, the role of NETs in thrombosis was tested in the *ex vivo* occlusive thrombus formation assay, where human whole blood was perfused through a capillary tube containing neutrophils treated with vehicle (resting neutrophils) or with PMA (to produce NETs). Preliminary results demonstrate that the formation of NETs reduced the mean time to occlusion from 40.4 ± 2.5 min to 24.9 ± 3.8 min (Fig. 6.4), suggesting that NETs exhibit a prothrombotic phenotype. When the blood was treated with the FXIIa inhibitor CTI, or the anti-FXI antibody 1A6 that inhibits FXIa generation as well as FXIa-mediated FIX activation, occlusion times were greater than 60 min regardless of the presence of NETs (Fig. 6.4). In contrast, an occlusive thrombus was observed after 48.2 ± 1.9 min in the presence of NETs when blood was incubated with the anti-FXI antibody 14E11 that selectively blocks FXIIa-mediated FXI activation but does not inhibit thrombin-mediated feedback activation of FXI. 14E11 prolonged the time to occlusion to over 60 min when NETs formation was not induced (Fig. 6.4). Lastly, the addition of an anti-TF blocking antibody prolonged the occlusion time by ~18 min as compared to the NETs surface alone (Fig. 6.4). Taken together, these results suggest that NETs activate coagulation in a TF-dependent manner, facilitating thrombin generation and feedback activation via activation of FXI, resulting in occlusive thrombus formation.

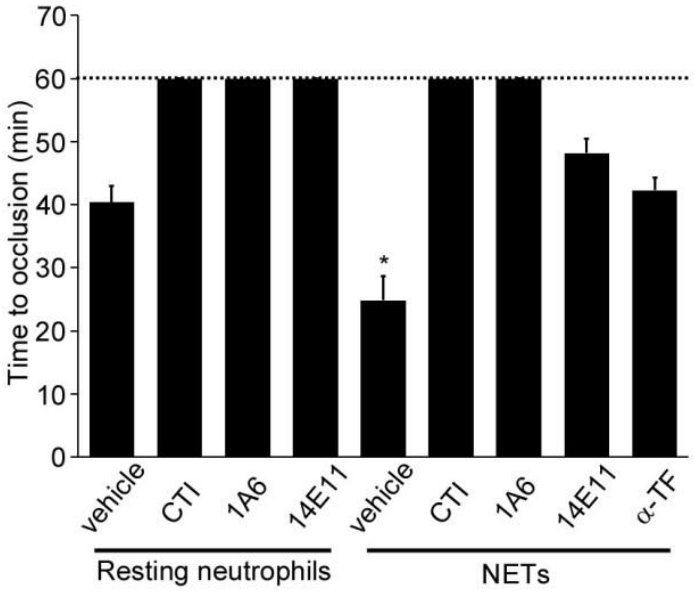

Figure 6.4 NETs promote occlusive thrombus formation.

Citrate anticoagulated human blood was recalcified and perfused through a surface of resting neutrophils or NETs in a glass capillary tube. Blood flow was driven by a constant pressure gradient and the time to occlusion of the tube was recorded. Blood was pretreated with CTI (20 μg/ml), 1A6 (10 μg/ml), 14E11 (10 μg/ml) or anti-TF (α-TF; 5 μg/ml). Data are mean ± SE (n = 3). **P* < 0.05, vs. resting neutrophils. Zoë Wong aided in generating these data.

As a next step, preliminary immunofluorescence experiments were performed to examine whether NETs serve as a platform for coagulation factor assembly in addition to exposing TF. Following the incubation of recalcified plasma over the NETs surface, both prothrombin and fibrin(ogen) were detected via immunofluorescence in proximity to extracellular DNA fibers (Fig. 6.4).

During thrombus formation, platelets have been shown to promote coagulation by exposing phosphatidylserine on their membrane surface, which in turn provides a platform for the assembly of coagulation factors such as prothrombin and fibrinogen (Berny *et al.* 2010). However, preliminary studies show that the blockade of phosphatidylserine binding sites with annexin V did not affect the localization of

prothrombin or fibrin(ogen) onto the NETs surface (Fig. 6.4). Moreover, the recruitment of prothrombin or fibrin(ogen) to the NETs surface was still observed in the presence of an anti-TF blocking antibody (Fig. 6.4), suggesting that the binding of prothrombin and fibrinogen to NETs is independent of the activation of the extrinsic coagulation pathway. Taken together, these preliminary data demonstrate that NETs promote the recruitment of coagulation factors in a distinct manner from platelets. Future experiments will be conducted to characterize the mechanisms by which NETs potentiate thrombus formation, including the role of NETs in the regulation of endogenous inhibitors of coagulation. In addition, the physical parameters of NETs-containing thrombus will be defined in a 3D environment under physiological levels of shear flow.

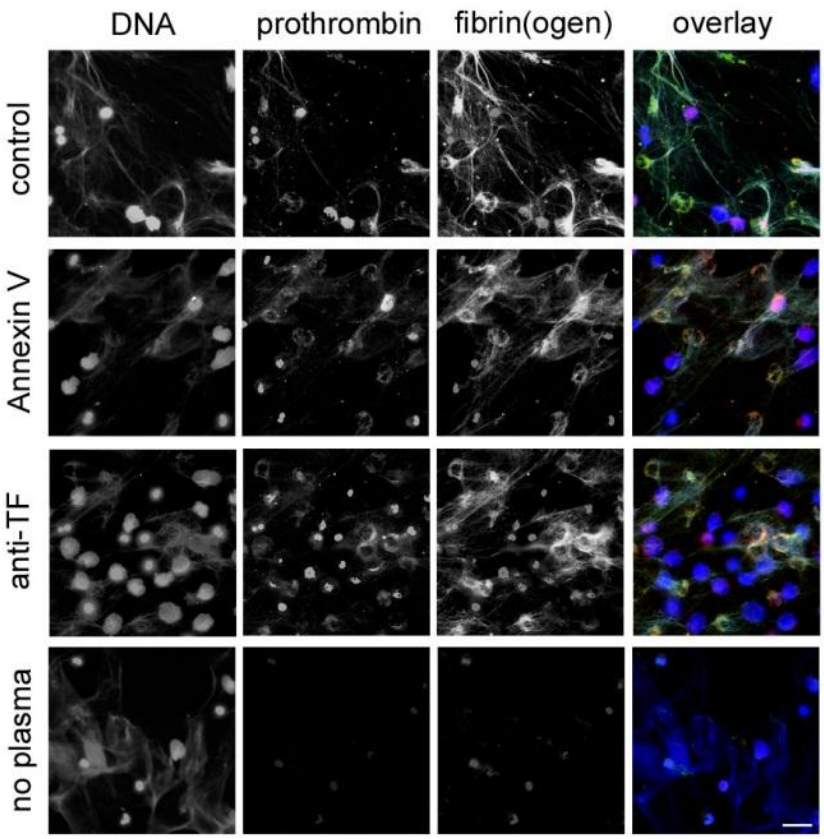

Figure 6.5 The binding of prothrombin and fibrin(ogen) on NETs.

NETs were induced by treating human neutrophils with PMA (100 nM) for 3 h, followed by incubation with human plasma in the presence of vehicle, annexin V or an anti-TF antibody for 15 min. Fixed samples were stained for prothrombin (red), fibrin(ogen) (green) and DNA (blue). The immunofluorescence specific for prothrombin or fibrin(ogen) was absent in the sample incubated without plasma (bottom). Scale bar = 20 μm.

6.3.2 Neutrophils in cancer progression

Clinical evidence suggests that elevated neutrophil count in the circulation is an independent marker of adverse prognosis in patients with cancer. While antitumor effects of neutrophils have been demonstrated, studies have indicated that a portion of neutrophils may facilitate cancer progression (Fridlender and Albelda 2012). However, the exact roles of neutrophils in cancer development and progression are still not fully described. Given that cancer-associated thrombosis, the second leading cause of death in

cancer patients, is prevalent in metastatic cancer, future studies will be designed to test the hypothesis that neutrophils contribute to the detrimental events underlying metastasis and cancer-associated thrombosis. In preliminary studies using a flow chamber system, experiments were performed to determine whether thrombus-bound neutrophils play a role in tumor cell recruitment and adhesion under shear. As shown in Fig. 6.6, cultured colon adenocarcinoma cells (SW620) tethered to and adhered downstream of thrombus-bound neutrophils (Fig. 6.6A). The presence of thrombus-bound neutrophils significantly increased the rate of SW620 interaction with thrombi (Fig. 6.6C). Overall, these results suggest that thrombus-bound neutrophils may augment the recruitment of circulating tumor cells and accelerate the progression of metastatic cancer.

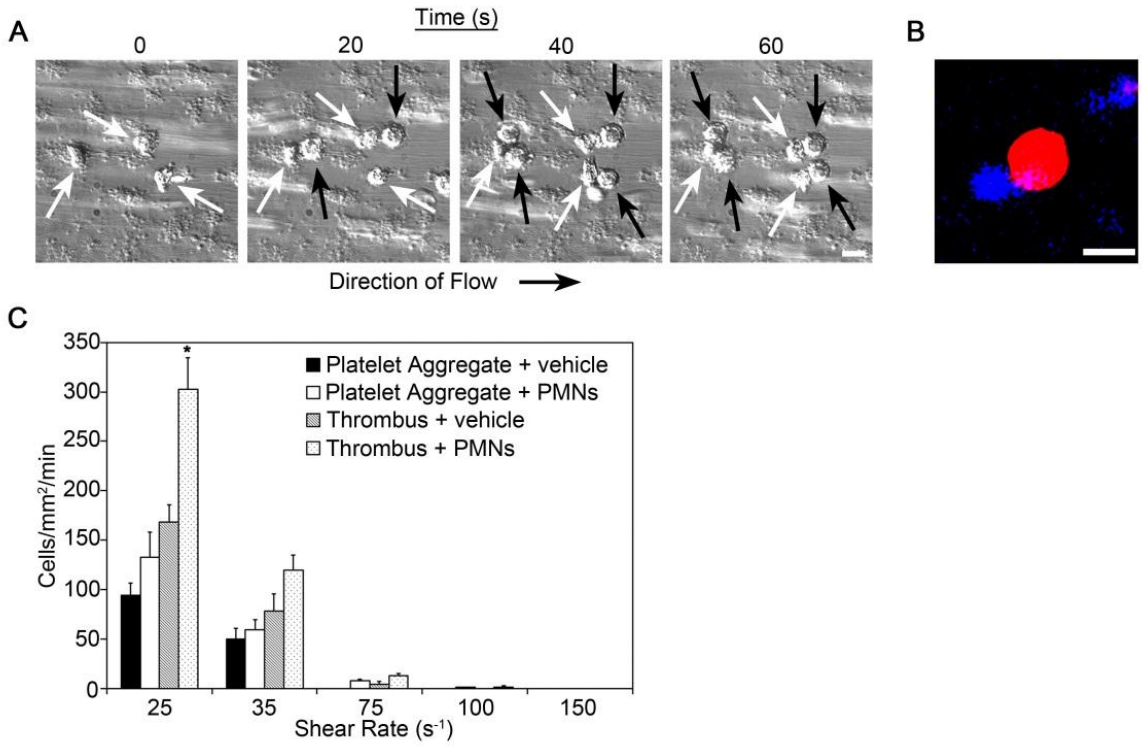

Figure 6.6 Neutrophils increase the rate of SW630 adenocarcinoma cell recruitment to formed platelet aggregates or thrombi under shear.

Platelet aggregates were formed by perfusing anticoagulated human blood, and thrombi were formed by perfusing recalcified human blood in a fibrinogen-coated glass capillary. Neutrophils (PMNs; blue) were perfused over formed platelet aggregates or thrombi prior to perfusion of SW620 cells (red) at the indicated shear rates. Surface interaction of SW620 cells (i.e. tethering, rolling, and firm adhesion) were monitored using fluorescence video capture. (A) Representative 40× DIC time-lapse images of neutrophils (white arrows) recruiting SW620 cells (black arrows) to formed thrombi. (B) Representative 20× fluorescent image of a SW620 cell (red) adhering to a neutrophil (blue). (C) The total rate of interacting SW620 cells to platelet aggregates or thrombi in the absence or presence of neutrophils. Data are represented as mean ± SEM of 3 independent experiments. *$P < 0.05$ as compared to the absence of neutrophils. Scale bar =10 μm. Flow chamber experiments were performed by Sandra Baker-Groberg.

Along these lines, Demers *et al.* have demonstrated that peripheral blood neutrophils display an increased sensitivity to NETs generation upon secondary stimulation in a murine model of chronic myelogenous leukemia as well as in solid tumor models (Demers *et al.* 2012). In a recent study by Cools-Lartigue *et al.*, infection-induced NETs have been shown to promote micrometastases by trapping circulating tumor cells (Cools-Lartigue *et al.* 2013). Collectively, it is possible that neutrophils in the malignant

microenvironment drive a vicious circle as follows; in the presence of a tumor, circulating neutrophils are predisposed to form NETs; once locally formed, NETs assist the recruitment of circulating tumor cells and allow their access to the secondary tissue; the exposure of NETs to blood induces thrombus formation; and thrombi further recruit peripheral neutrophils and metastatic cells.

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Biographical Sketch

Asako Itakura was born on December 22, 1986 in Tokyo, Japan to Takashi and Yuko Itakura.

Asako attended Meguro Seibi Gakuen High School and, upon graduation, enrolled at the University of Tokyo. During her undergraduate studies, she joined the laboratory of microbiology with her advisors, Drs. Manabu Arioka and Katsuhiko Kitamoto. She was awarded the Young Investigator Award at the Japan Society for Bioscience, Biotechnology and Agrochemistry Kanto meeting, for her undergraduate research project. In March of 2009, she earned her Bachelor of Science degree in Agriculture.

Asako continued her education at Oregon Health & Science University (OHSU), joining the laboratory of Dr. Owen McCarty in the Department of Cell & Developmental Biology in January 2010. She received a Bayer Schering Pharma AG International Fellow Award to conduct her research in the laboratory of Dr. Christoph Gerdes at Bayer Pharma in Wuppertal, Germany. Asako returned from Europe to finish her graduate research centered on the regulatory mechanisms of neutrophil functions.

During her graduate studies at OHSU, Asako was awarded an ASH Abstract Achievement Award and received research funding through a Vertex scholarship. She has presented her research in peer-reviewed journals and at international conferences throughout the U.S., Asia and Europe. Current publications and presentations are listed below:

Publications

1. **Itakura A**, Aslan JE, Sinha S, White-Adams TC, Patel IA, Meza-Romero R, Vandenbark AA, Burrows GG, Offner H, McCarty OJ. "Characterization of human platelet binding of recombinant T cell receptor ligand." *J Neuroinflammation*. 2010 Nov 8;7:75.
2. Nie Z, Scott GD, Weis PD, **Itakura A**, Fryer AD, Jacoby DB. "Role of TNF-α in virus-induced airway hyperresponsiveness and neuronal M_2 muscarinic receptor dysfunction." *Br J Pharmacol*. 2011 Sep;164(2b):444-52.

3. **Itakura A**, Verbout NG, Phillips KG, Insall RH, Gailani D, Tucker EI, Gruber A, McCarty OJ. "Activated factor XI inhibits chemotaxis of polymorphonuclear leukocytes." *J Leukoc Biol*. 2011 Nov;90(5):923-7.

4. Winkler CW, Foster SC, Matsumoto SG, Preston MA, Xing R, Bebo BF, Banine F, Berny-Lang MA, **Itakura A**, McCarty OJ, Sherman LS. "Hyaluronan anchored to activated CD44 on central nervous system vascular endothelial cells promotes lymphocyte extravasation in experimental autoimmune encephalomyelitis." *J Biol Chem*. 2012 Sep 28;287(40):33237-51.

5. Freeman ML, Burkum CE, Lanzer KG, Roberts AD, Pinkevych M, **Itakura A**, Kummer LW, Szaba FM, Davenport MP, McCarty OJ, Woodland DL, Smiley ST, Blackman MA. "Gammaherpesvirus latency induces antibody-associated thrombocytopenia in mice." *J Autoimmun.* 2013 May;42:71-9.

6. Winkler CW, Foster SC, **Itakura A**, Matsumoto SG, Asari A, McCarty OJ, Sherman LS. "Hyaluronan oligosaccharides perturb lymphocyte slow rolling on brain vascular endothelial cells: Implications for inflammatory demyelinating disease." *Matrix Biol*. 2013 Apr 24;32(3-4):160-8.

7. Wuhanqimuge, **Itakura A**, Matsuki Y, Tanaka M, Arioka M. "Lysophosphatidylcholine enhances NGF-induced MAPK and Akt signals through the extracellular domain of TrkA in PC12 cells." *FEBS Open Bio.* 2013 May 30;3:243-51.

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9. **Itakura A** and McCarty OJ. "Pivotal role for the mTOR pathway in the formation of neutrophil extracellular traps (NETs) via regulation of autophagy." *Am J Physiol Cell Physiol*. 2013 Aug;305(3):C348-54.

10. Aslan JE, Baker SM, Haley KM, Loren CP, **Itakura A**, Pang J, Greenberg DL, David LL, Chernoff J, Manser E, McCarty OJ. "The PAK system links Rho GTPase signaling to thrombin-mediated platelet activation." *Am J Physiol Cell Physiol*. 2013 Sep;305(5):C519-28.

11. Baker-Groberg SM, **Itakura A**, Gruber A, McCarty OJ. "Role of coagulation in the recruitment of colon adenocarcinoma cells to thrombus under shear." *Am J Physiol Cell Physiol*. 2013 Jul 31 [Epub ahead of print]

12. **Itakura A**, Aslan JE, Kusanto BT, Phillips KG, Porter JE, Newton PK, Nan X, Insall RH, Chernoff J, McCarty OJ. "p21-activated kinase (PAK) regulates cytoskeletal reorganization and directional migration in human neutrophils." *PLoS ONE* 2013; 8(9): e73063.

13. Aslan JE, Phillips KG, Healy LD, **Itakura A**, Pang J, McCarty OJ. "Histone deacetylase 6 (HDAC6)-mediated deacetylation of α-tubulin coordinates cytoskeletal and signaling events during platelet activation." *Am J Physiol Cell Physiol*. 2013 Sep 11. [Epub ahead of print]

Book Chapters

1. Aslan JE, **Itakura A**, Gertz JM, McCarty OJ. "Platelet shape change and spreading." *Methods Mol Biol.* 2012;788:91-100.

Presentations

1. **Itakura A**, Aslan JE, Sinha S, White-Adams TC, Patel IA, Vandenbark AA, Burrows GG, Offner H, McCarty OJ. "Characterization of human platelet binding of recombinant T cell receptor ligand" XIth European Symposium on Platelet and Granulocyte Immunobiology, Beaune, France (Oct, 2010).
2. **Itakura A**, White-Adams TC, Verbout NG, Gailani D, Gruber A, McCarty OJ. "Interaction of neutrophil granulocytes with coagulation factors XI and XII" XIth European Symposium on Platelet and Granulocyte Immunobiology, Beaune, France (Oct, 2010).
3. **Itakura A**, Verbout NG, Phillips KG, Insall RH, Gailani D, Tucker EI, Gruber A, McCarty OJ. "Activated factor XI (FXIa) inhibits chemotaxis and oxidative burst of polymorphonuclear leukocytes" XXIII Congress of the International Society on Thrombosis and Haemostasis, Kyoto, Japan (July, 2011).
4. **Itakura A**, Aslan JE, Kusanto BT, Phillips KG, Insall RH, Chernoff J, McCarty OJ. "p21-activated kinases regulate directional migration and cytoskeletal organization in human neutrophils" Annual Meeting of the American Society of Hematology, Atlanta, GA (Dec, 2012)**.**
5. **Itakura A** and McCarty OJ. "Pivotal role for the mTOR pathway in the formation of neutrophil extracellular traps (NETs) via regulation of autophagy." XXIV Congress of the International Society on Thrombosis and Haemostasis, Amsterdam, Netherlands (July, 2013).

Manuscript reviewer

1. Immunology
2. PLOS ONE

Undergraduate Research Mentor

1. Branden Kusanto (2012-2013): currently undergrad OSU Chemical Engineering
2. Mentor to Branden Kusanto, Bio & Pharmaceuticals Poster Award, AIChE National Meeting, 2012
3. Mentor for an American Heart Association Undergraduate Research Fellowship to Branden Kusanto, 2013