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MLK3 regulates fMLP-stimulated neutrophil motility

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Abstract

Introduction—Mixed Lineage Kinase 3 (MLK3) is part of the intracellular regulatory system that connects extracellular cytokine or mitogen signals received through G-protein coupled receptors to changes in gene expression. MLK3 activation stimulates motility of epithelial cells and epithelial-derived tumor cells, but its role in mediating the migration of other cell types remains unknown. Since neutrophils play a crucial role in innate immunity and contribute to the pathogenesis of several diseases, we therefore examined whether MLK3 might regulate the motility of mouse neutrophils responding to a chemotactic stimulus, the model bacterial chemoattractant fMLP.

Methods—The expression of *Mlk3* in mouse neutrophils was determined by immunocytochemistry and by RT-PCR. *In vitro* chemotaxis in a gradient of fMLP, fMLP-stimulated random motility, fMLP-stimulated F-actin formation were measured by direct microscopic observation using neutrophils pre-treated with a novel small molecule inhibitor of MLK3 (URMC099) or neutrophils obtained from *Mlk3*−/− mice. *In vivo* effects of MLK3 inhibition were measured by counting the fMLP-induced accumulation of neutrophils in the peritoneum following pre-treatment with URMC099 in wild-type C57Bl/6 or mutant *Mlk3*−/−mice.

Results—The expression of *Mlk3* mRNA and protein was observed in neutrophils purified from wild-type C57Bl/6 mice but not in neutrophils from mutant *Mlk3*−/− mice. Chemotaxis by wild-type neutrophils induced by a gradient of fMLP was reduced by pre-treatment with URMC099. Neutrophils from C57Bl/6 mice pretreated with URMC099 and neutrophils from *Mlk3*−/− mice moved far less upon fMLP-stimulation and did not form F-actin as readily as untreated neutrophils from C57Bl/6 controls. *In vivo* recruitment of neutrophils into the peritoneum by fMLP was

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significantly reduced in wild-type mice treated with URMC099, as well as in untreated *Mlk3*−/− mice – thereby confirming the role of MLK3 in neutrophil migration.

Conclusions—*Mlk3* mRNA is expressed in murine neutrophils. Genetic or pharmacologic inhibition of MLK3 blocks fMLP-mediated motility of neutrophils both *in vitro* and *in vivo*, suggesting that MLK3 may be a therapeutic target in human diseases characterized by exuberant neutrophil migration.

Keywords

Mixed lineage kinase 3; neutrophil; chemotaxis; mouse; MLK3

1. Introduction

Mixed Lineage Kinase 3 (MLK3, also known as MAP3K11) is a widely expressed, 93 kDa serine/threonine kinase that serves as part of a phosphorelay system linking mitogen or cytokine-activated G-protein coupled receptors (GPCR) to altered nuclear gene expression (Fortin et al., 2013) and other cell functions. Activation of the MLK3 kinase requires the direct interaction of cdc42 with MLK3's cdc42/Rac-interactive binding (CRIB) motif resulting in the displacement of MLK3's autoinhibitory Src homology 3 (SH3) domain subsequently facilitating two steps in kinase activation: autophosphorylation of Thr277 and Ser281 and dimerization via MLK3's leucine zipper domain (Gallo and Johnson, 2002; Handley et al., 2007; Leung and Lassam, 1998). Once activated, MLK3 in turn activates its substrates, including the mitogen-activated kinase kinases MKK4/7 and MKK3/6, which then phosphorylate the MAP kinases JNK, ERK or p38, depending on the cell type.

In addition to its interactions with the Rho GTPases and its immediate substrates, MLK3 has been observed to interact with numerous intracellular regulatory and structural molecules including the MAPK scaffold proteins JIP-1 and JIP-2 (Nihalani et al., 2001), the cell cycle regulator prolyl isomerase Pin-1 (Rangasamy et al., 2012), centrosomes and microtubules (Swenson et al., 2003), the kinase AKT (Barthwal et al., 2003; Zhang et al., 2006), TNF-receptor-associated factor 2 (Mishra et al., 2010), Hsp90 (Zhang et al., 2004) and the feedback regulator p63RhoGEF (Swenson-Fields et al., 2008).

Because MLK3 has such a diverse array of interactions, it has been proposed that MLK3 may be a biochemical integrator of multiple signaling pathways (Swenson-Fields et al., 2008). MLK3 has been implicated in essential functions in normal and transformed cells including cell division (Rangasamy et al., 2012; Swenson et al., 2003), apoptosis (Kim et al., 2004; Mota et al., 2001; Xu et al., 2001), and differentiation (Brancho et al., 2005; Cowley et al., 1994).

A major result of MLK3 activation is the stimulation of cell motility and migration which has been observed in several cell types *in vivo* and *in vitro* (Huang et al., 2004). For example, the migration of human gastric cancer cells (Mishra et al., 2010) and the migration and invasiveness of human breast cancer cells were reduced by pretreatment with *Mlk3*-specific siRNA or the small molecule MLK inhibitor CEP1347 (Chen and Gallo, 2012). In another recent report, the *in vivo* healing of jejunal ulcers, dependent on migration of intestinal epithelial cells, was reduced in *Mlk3*−/− mice compared to wild-type mice and this was mirrored by the reduced migration of Caco-2 cells *in vitro* following treatment with an MLK inhibitor (Kovalenko et al., 2012).

The role of MLK3 in eliciting movement of primary epithelial cells and tumor cells putatively derived from epithelial cells (gastric, breast cancer cells) suggested that it might play a common role in other cell types that migrate in response to environmental cues –

including innate immune cells such as neutrophils. This is potentially important since the excessive accumulation of neutrophils in certain pathologic conditions such as sepsis, ischemia reperfusion injury, respiratory distress syndrome, and multi-organ failure (Amulic et al., 2012; Mahmudi-Azer and van Eeden, 2003; Wright et al., 2010) - raising the prospect of therapeutic modulation of MLK3 activity for accelerating or delaying neutrophil migration.

To examine the role of MLK3 in neutrophil motility, we took advantage of a new small molecule MLK3 inhibitor, URMC099, which has been shown to be neuro-protective in *in vitro* and *in vivo* models for HIV-associated neurocognitive disorders (Marker et al., 2013). In this paper, we demonstrate that MLK3 regulates short-term events in the induction of motility of neutrophils responding to the potent chemoattractant fMLP. Further, the observed phenotype of reduced responsiveness in neutrophils derived from *Mlk3*−/− mice was replicated by pre-treatment of neutrophils from wild-type mice with URMC099.

2. Materials and Methods

2.1 Reagents

Recombinant Protein A (rProtein A) was purchased from Invitrogen (Cat. No. 101100). Recombinant Intercellular Adhesion Molecule 1 (ICAM-1, also called CD54) expressed as a chimera with human IgG1 Fc (rICAMFc) was purchased from R&D Systems (Minneapolis, MN, Cat. No. 796-IC-050). N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) was purchased from Sigma-Aldrich (Cat No. F3506); Rat monoclonal antibodies specific for mouse neutrophils, Ly-6G-phycoerythrin (PE, Cat. No. 551461) and Ly-6G-allophycocyanin (APC, Cat. No. 560599) were obtained from BD Pharmingen. Fluorescein-conjugated phalloidin was purchased from Life Technologies (Cat. No. F432). Rat anti-mouse Fc-Receptor antibody 2.4G2 was obtained from Life Technologies (Cat. No. 553141). Goat anti-MLK3 antibody was purchased from Santa Cruz Biotechnology (Cat. No. sc-15068), and Alexa Fluor® 488-conjugated donkey anti-goat IgG (H+L) was obtained from Invitrogen (Cat. No.A-11055).

The MLK3 inhibitor URMC099 was synthesized by and obtained from Califia Bio (Goodfellow et al., 2013b; Marker et al., 2013).

2.2 Experimental animals

Eight week old male C57Bl/6 mice were purchased from Charles River Laboratories and used before they were twelve weeks old. *Mlk3*−/− mice were back-crossed to C57Bl/6 animals (Jackson Laboratories) for ten generations and maintained as a homozygous strain (Brancho et al., 2005); animals were kindly provided by Dr. Roger Davis (University of Massachusetts Medical School). Mice were maintained on an *ad libitum* diet, with 12 hours light cycle. All procedures were reviewed and approved by the University of Rochester Committee on Animal Resources.

2.3 Murine neutrophils

Murine neutrophils were harvested from the humeri and femurs from freshly sacrificed male C57Bl/6 and *Mlk3*−/− mice. Neutrophil-rich cell populations were extruded from the medullary canals of the excised bones, washed in Red Blood Cell Lysis Buffer (Gibco), passed through a 70 μm cell strainer, resuspended in PBS and maintained at 4°C.

For motility and chemotaxis assays, neutrophils were positively selected for expression of a neutrophil-specific cell surface marker, Ly-6G, using Anti-Ly-6G MicroBead Kit (Miltenyi, Cat. No. 130-092-332) according to the manufacturer's instructions. Purity of neutrophil

preparations was measured by concentrating the cells using a Cytospin and staining with xanthene and thiazine dyes using the Diff-Quik Staining Set (Siemens, Cat. No. B4132-1A).

2.4 Neutrophil abundance in the bone marrow and blood

Cytospin preparations of cells harvested from the bone marrow of three C57Bl/6 and three *Mlk3*−/− mice were stained as in section 2.3. Neutrophils and total lymphocytes were counted. Results are expressed as the percentage of total bone marrow cells that are neutrophils or neutrophil precursors as defined by their distinctive nuclear morphologies.

To determine the abundance of neutrophils in blood, 100 μL of blood was harvested by retro-orbital bleeding from C57Bl/6 and *Mlk3*−/− mice using EDTA as the anticoagulant. Differential cell counting was performed using a Heska HemaTrue® Veterinary Hematology Analyzer. Results for neutrophils are reported as the mean and standard deviation of neutrophils/μL of blood for five mice of each type.

To determine the effect of short-term exposure to URMC099, the same mice were injected I.P. with URMC099, 10 mg/kg. After 60 minutes, blood was sampled and neutrophils counted as above.

2.5 Viability of neutrophils in the presence of URMC099

Two measures were used to assess the short-term toxicity of URMC099 for purified neutrophils from C57Bl/6 and *Mlk3*−/− mice. The first was the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Cat. No. G5421) which measures the reduction of the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS) to a formazan by the mitochondria of living cells. The second was a measure of cell death: the leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH), as measured using the Sigma Lactic Dehydrogenase-based *In Vitro* Toxicology Assay Kit (Sigma, Cat. No. TOX-7).

Briefly, freshly isolated neutrophils were distributed into the wells of a 96-well plate at a density of 10^5 cells/well. URMC099 (or vehicle alone) was then added to a final concentration of 100 nM and the cells were incubated at 37°C for 60 minutes. Each culture was then split and assayed for cell viability with CellTiter MTS kit or cell death with the LDH kit, according to the manufacturer's instructions.

2.6 Immunocytochemical detection of MLK3 in isolated neutrophils

Cytospin preparations of purified neutrophils from C57Bl/6 and *Mlk3*−/− mice were permeabilized with BD Cytofix/Cytoperm™ for 10 minutes and washed once with BD PermWash Wash Buffer. Slides with fixed and permeabilized cells were then stained 24 hours at 4°C with goat anti-MLK3 antibody (Santa Cruz Biotechnology, Cat. No. sc-15068), a polyclonal antibody which recognizes the N-terminus of murine MLK3. After three washes with BD PermWash Wash Buffer, the cells were incubated for 2 hours at room temperature with the secondary antibody, Alexa Fluor® 488-conjugated donkey anti-goat IgG (H+L) (Invitrogen, Cat. No.A-11055), and 1 μg/ml of the DNA-specific dye Hoechst 33342. Following three wash steps, stained slides were mounted with Prolong Gold mounting medium (Invitrogen) and images were recorded using an Olympus FV1000 laser scanning confocal microscope.

2.7 Measurement of *Mlk3* cDNA expression in neutrophils

Expression of *Mlk3 mRNA* in isolated neutrophils was measured by RT-PCR using validated *Mlk3*-specific PCR primers. Total RNA was isolated from C57Bl/6 and *Mlk3*−/− neutrophils using the Invitrogen PureLink® kit (Cat. No. 12183-018) following the manufacturer's

instructions. After DNase treatment to remove potentially contaminating DNA and determination of RNA concentration by A_{260} using NanoDrop, two μg total RNA was used as a template for cDNA synthesis using Superscript III (Invitrogen, #18080-044) in a 20 μL reaction volume according to manufacturer's instructions. *Mlk3* cDNA was then amplified by PCR using primers that span exons 1 and 2 (TaqMan assay #Mm01233534, Invitrogen, Cat. No. 4351372). As a positive control, the expression of mRNA encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using validated primers (Taqman assay #Mm99999915, Invitrogen, Cat. No. 4331182).

The resulting products were separated on 2% agarose gels and detected by staining with ethidium bromide. Samples were considered positive when a PCR product of the predicted size was detected after 40 cycles of PCR.

2.8 Chemotaxis of murine neutrophils in a gradient of the chemoattractant fMLP

Directional movement was measured by placing neutrophils in a linear gradient of fMLP and measuring their Accumulated Distance (total distance traversed in the 20 minute observation period) and Forward Moving Index (the ratio of movement in the direction of the fMLP gradient to the Accumulated Distance). Pre-treatment of the neutrophils, establishment of the fMLP gradient and observation of neutrophil movement were all performed at 37°C in μ-Slide Chemotaxis chambers pre-coated with Collagen IV (Ibidi Integrated Diagnostics, Cat. No. 80302) or in plain Ibidi slides (Ca. No. 80306) coated with ICAM-1 as described in section 2.9. Analysis of chemotaxis was performed using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012.) supplemented with the Manual Tracking plug-in by Fabrice Cordelières, Institut Curie, Orsay, France (http://rsbweb.nih.gov/ij/plugins/track/track.html).

Briefly, freshly isolated neutrophils were washed and resuspended at a concentration of 3×10^3 cells/μL in cold Hank's Buffered Salt Solution (HBSS) supplemented with 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2 % BSA (Lecut et al., 2009). Neutrophils were placed in the viewing chamber by adding 6 μL of the cell suspension and allowed to adhere for 20 minutes. Neutrophils attached in the viewing area were then washed with HBSS and then incubated with 100 nM URMC099 or vehicle only (0.01% DMSO) in HBSS for 15 minutes. The fMLP gradient was established by adding 18 μL of 2500 nM fMLP so that on the high side of the viewing chamber the concentration was 400 nM and on the low side it was 0 nM. After 15 minutes, a linear gradient was established and observation was initiated by recording the movement every minute for twenty minutes. For the analysis, 30–40 neutrophils were randomly selected on the first image and then tracked through 20 images spanning twenty minutes.

Two attributes of fMLP-induced cell movement were measured. Accumulated Distance (AD) is the total distance traversed by the center of each cell obtained by adding the distance moved in successive one-minute intervals throughout the observation period (essentially the line integral). The Chemotactic Index is the net movement in the direction of the fMLP gradient divided by the AD (Heit et al., 2008). Each measurement is the average of ≥ 30 cells from 4–6 separate experiments (≥ 120 neutrophils).

2.9 fMLP-stimulated motility of neutrophils

Neutrophil migration was determined by measuring the distance traveled on an ICAM-1-coated surface following exposure to the chemoattractant fMLP (Elphick et al., 2009). Glass microscope slides were first incubated with rProtein A (50 μg/mL in Tris-buffered saline, pH 9.0 (TBS)), 37°C for 60 minutes, washed with TBS, and then incubated with rICAMFc

(5 μg/mL in TBS), RT for 120 minutes and then washed with phosphate-buffered saline (PBS), pH 7.4.

Freshly isolated murine bone marrow neutrophils (100 μL, 10^6 cells/mL) were added to 2 mL of L15 medium supplemented with 2 mM glucose, placed on the ICAM-coated slide and incubated at 37°C for 20 minutes to allow the cells to bind to the ICAM-coated surface. ICAM-bound neutrophils were then incubated 20 minutes at 37°C with L15 medium containing 2 mM glucose and supplemented with potential inhibitors or vehicle alone. Cells were then imaged for 10 minutes at 37°C to determine the level of migration without stimulation. Then fresh media containing fMLP was added to the cells at a final concentration of 1 μM fMLP and imaging was initiated immediately for an additional 10 minutes to measure the fMLP-stimulated level of migration.

The migration of neutrophils was monitored using 10X magnification on an inverted microsope capturing images every ten seconds. For each condition, twenty randomly selected cells were tracked for ten minutes and the distance each migrated was calculated using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012.). Results are presented as the dimensionless Motility Index which is the ratio of the total distance moved by neutrophils in the presence of fMLP divided by total distance migrated in the presence of vehicle alone. Each measurement is the mean and standard deviation from three replicates of each condition.

2.10 F-actin formation in fMLP-activated neutrophils

The formation of F-actin was detected by its interaction with fluorescently labeled phalloidin. Freshly isolated bone marrow cells from C57Bl/6 or *Mlk3*−/− mice were stained with phycoerythrin-conjugated monoclonal anti-murine Ly-6G antibody (PE-Ly6G) which specifically labels neutrophils in the presence of an excess of anti-FcR monoclonal antibody 2.4G2. After incubation on ice for 30 minutes, the bone marrow cells were washed with ice-cold HBSS, and then transferred into the wells of a 24-well microculture plates containing pre-warmed L15 medium containing 2 mM glucose, the activator (fMLP) and the inhibitors at the test concentrations. After incubation at 37°C for 2 minutes, samples from each condition were fixed by mixing with an equal volume of 4% paraformaldehyde and incubation for 5 minutes at RT, and then permeabilized by the addition of BD PermWash™ (BD Cat. No. 51-2091KZ) for 20 minutes at 4°C. Prepared cells were then incubated with 2 U/mL FITC-conjugated phalloidin in BD PermWash™ for 15 minutes to selectively stain F-actin.

Images were taken of five fields per well. Each condition was done in duplicate, so each measurement is the result of counting ten fields each containing an average of 25 neutrophils or a total of 250 cells. Neutrophils (cells with red perimeter staining by PE-anti-Ly6) were scored as F-actin positive (green, extranuclear, ring fluorescence) and F-actin negative (uniform weak green fluorescence). Data are presented as the percentage of neutrophils that displayed ring fluorescence indicative of F-actin formation.

2.11 Measurement of fMLP-induced recruitment of neutrophils to the peritoneal cavity

C57Bl/6 or *Mlk3*−/− mice were pretreated by intraperitoneal injection of 10 mg/kg URMC099 (2 mg/mL solution in 5%DMSO/42.5% PEG/52.5% saline) or vehicle alone. After 60 minutes, the mice received an intraperitoneal injection of fMLP (50 μg in 100 μL saline) to stimulate neutrophil-rich peritoneal exudates. After 30 minutes, the animals were euthanized by CO_2 asphyxiation and the peritoneal cavities were washed with 4 mL of RPMI with 10% FBS containing 3 mM EDTA and 10 U/ml heparin. The recovered cells

were stained with the neutrophil-specific monoclonal antibody APC-Ly6G for 30 minutes at 4 °C in the presence of the FcR-blocking monoclonal antibody 2.4G2. Stained and washed peritoneal exudate cells were resuspended in 1.0 mL PBS to which 2.5 μL of CountBright™ 7μm standardization beads (Invitrogen, Cat. No. C36950) was added to provide an internal reference for flow cytometry.

The total number of neutrophils recruited into the peritoneum was measured by counting APC-Ly6G positive cells by flow cytometry, normalizing to the number of internal CountBright™ reference particles and adjusting for the total volume recovered from the washing of the peritoneal cavity. Results are reported as the average ± the standard deviation of the total number of Ly6G-positive cells per mouse; 3–5 mice were used for each condition.

2.12 Statistical analysis

Mean values were compared by two-tailed *t*-tests using Microsoft Excel function. Differences were considered significant when *p* values were less than 0.05.

3. Results

3.1 The abundance and viability of neutrophils is not altered by genetic MLK3 deficiency or by exposure to the MLK3 inhibitor URMC099

In order to examine the role of MLK3 in neutrophil activation, it was first necessary to rule out unintended effects of the genetic and pharmacologic interventions we planned to utilize. We first measured the relative abundance of neutrophils in bone marrow of C57Bl/6 and *Mlk3*−/−mice and found it to be essentially identical: neutrophils comprised 39 ± 9% of bone marrow cells in C57Bl/6 mice and 41 ± 16% in *Mlk3*−/− mice (mean + standard deviation, calculated from 5 mice of each type).

We then assessed the purity of neutrophils that were prepared by magnetic separation using the neutrophil marker Ly-6G. Neutrophils harvested from the bone marrow of C57Bl/6 and *Mlk3*−/− mice were purified, collected by Cytospin, stained with DiffQuick and examined by light microscopy (Fig. 1). Greater than 99% of the cells displayed the signature staining of murine bone immature neutrophils and promyelocytes (Eash et al., 2009; Olins and Olins, 2005; Sukhumavasi et al., 2007). No difference was observed in neutrophils prepared from C57Bl/6 or *Mlk3*−/− mice. Based on the purity of these cells, we felt confident that we could measure *Mlk3* gene expression and attribute it to neutrophils (section 3.2).

The absence of a significant difference in neutrophil abundance in the bone marrow between the C57Bl/6 and *Mlk3*−/− mice suggested that there was no defect in neutrophil development in the bone marrow of the knockout mice. We next enumerated the neutrophils in the blood of C57Bl/6 and *Mlk3*−/− mice to assess neutrophil abundance in the circulation. Whole blood samples were collected by retro-orbital bleeding and cell types were enumerated using an automated cell counter. The abundance of neutrophils was essentially identical: $0.95 \pm 0.25 \times 10^3/\mu L$ in C57Bl/6 and $1.25 \pm 0.20 \times 10^3/\mu L$ in *Mlk3*−/− mice (mean + standard deviation, calculated from 5 mice of each type). In addition, the abundance of circulating neutrophils was unaltered in mice pre-treated for 60 minutes with URMC099: 1.20 ± 0.25 x$10^3/\mu L$ in C57Bl/6 and 1.00 ± 0.15 x$10^3/\mu L$ in *Mlk3*−/− (mean ± standard deviation, calculated from 5 mice of each type). These numbers are consistent with those reported for male C57Bl/6 mice (Flurkey, 2009). Finally, no differences between C57Bl/6 and *Mlk3*−/− mice were observed in the relative abundance of other cell types in the blood (data not shown).

To directly test for short term toxicity due to URMC099, freshly isolated neutrophils from C57Bl/6 and *Mlk3*−/− mice were cultured for 60 minutes at 37°C in the presence of 100 nM URMC099 or vehicle. Cell viability was then assessed by the MTS reduction assay and cell death was measured by the release of LDH. The levels of MTS reduction and LDH activity were identical in all four conditions: C57Bl/6 or *Mlk3*−/− neutrophils incubated with 100 nM URMC099 or vehicle. No effect of URMC099 on neutrophil viability was observed.

3.2 *Mlk3* is expressed in neutrophils from C57Bl/6 mice and not in neutrophils from Mlk3−/−mice

Even though it has been generally assumed (Hakkim et al., 2011; Selvatici et al., 2006; Tsai et al., 2013; Zhong et al., 2003), the expression of *Mlk3* has not been directly demonstrated in neutrophils. To provide direct evidence of MLK3 expression, we performed immunocytochemistry using a polyclonal goat antibody that recognizes the N-terminus of murine MLK3 with the expectation that neutrophils from C57Bl/6 mice would be distinctly positive while those from the *Mlk3*−/− mice would be negative. The resulting images presented in Fig. 2A, depict neutrophils recognizable by their toroidal shape in the DIC images (left) and the distribution of DNA in the DAPI-stained images (center). Neutrophils from C57Bl/6 mice are brightly stained for anti-MLK3 over their entire area while those from *Mlk3*−/− mice were stained in a weak, stippled pattern. The weak staining of the *Mlk3*−/− neutrophils may be explained by non-specific binding or by cross-reactivity with other MAP3K family members.

To provide an additional line of direct evidence for *Mlk3* expression, we detected *Mlk3* mRNA in freshly isolated neutrophils by RT-PCR. The characteristic 79 bp RT-PCR product was present in neutrophils purified from C57Bl/6 mice and it was not detected in neutrophils from *Mlk3*−/− mice (Fig. 2B). As a control, mRNA encoding the glycolytic enzyme GAPDH was detected in neutrophils from the wild-type C57Bl/6 mice and the knock-out mutant *Mlk3*−/− mice.

3.3 Inhibition of MLK3 by URMC099 reduces cell motility and chemotaxis induced in neutrophils by a gradient of fMLP

To test role of MLK3 in neutrophil motility, we initially tested the effect of the MLK3-specific inhibitor URMC099 (Marker et al., 2013) on the *in vitro* stimulation of motility by the well characterized neutrophil chemoattractant fMLP. We first tested whether inhibition of MLK3 would restrict chemotaxis in an fMLP gradient. Following pre-treatment with either 100 nM URMC099 or vehicle only, neutrophils were placed in μ-Slide Chemotaxis chambers pre-coated with Collagen IV either in the absence of fMLP or in a gradient of fMLP ranging from 0 to 400 nM. The Accumulated Distance (AD) of each cell was measured in addition to the net movement in the direction of the fMLP gradient. As a measure of directional movement, the Chemotactic Index was calculated as the ratio of the net movement in the direction of the gradient and the AD. Representative data is shown in Fig. 3A and 3B.

In the gradient of fMLP (0–400 nM), neutrophils moved twice as far as vehicle-treated neutrophils (63 μm compared to 30 μm) and this increase in AD was blocked by the presence of 100 nM URMC099 (Fig. 3C). Similarly, directional movement, reflected in the Chemotactic Index, increased from essentially zero (0 ± 0.03) in the absence of fMLP to 0.18 ± 0.03 in the fMLP gradient and this effect was eliminated by pre-treatment with URMC099 where the Chemotactic Index was 0.03 ± 0.03 (Fig. 3D). Essentially identical data were obtained using μ-Slide Chemotaxis chambers coated with ICAM-1 (data not shown).

3.4 The MLK3-specific inhibitor URMC099 reduces fMLP-induced motility by neutrophils in a dose-dependent manner

To examine the inhibition of MLK3 activity by URMC099 in more detail, we used an assay that measured only the fMLP-induced enhancement of motility and measured the dose dependence of URMC099. Freshly isolated and purified bone marrow neutrophils from male C57Bl/6 mice were placed on an ICAM-1-coated surface and cultured for 20 minutes with medium containing URMC099 or vehicle alone. Unstimulated movement was observed over a ten-minute span. Then 1.0 μM fMLP was added and neutrophil migration was observed for an additional ten minutes.

Unstimulated neutrophils typically migrated only a few μm whereas most of the fMLP-stimulated cells moved multiple cell lengths averaging over tens of μm (Fig. 4B). Values of the Motility Index, the ratio of distance moved by fMLP-stimulated cells to that migrated by unstimulated cells, varied among experiments due to the degree of activation in the unstimulated population.

Neutrophils pre-treated with URMC099 at concentrations ranging from 0.2 to 200 nM resulted in a dose-dependent reduction in Motility Index (Fig. 4A); fMLP-stimulation of migration was completely blocked by URMC099 at 100 nM, consistent with the reported value for the K_i of URMC099 for MLK3 kinase activity which is less than 0.1 μM (Marker et al., 2013).

3.5 fMLP-induced motility is impaired in neutrophils from Mlk−/− mice

The inhibition of fMLP-induced motility by URMC099 strongly implicates MLK3 in signal transduction and specifically predicts that *Mlk3* knockout mice will exhibit a similar inhibition. To test this hypothesis, non-activated neutrophils were purified from the bone marrow of male C57Bl/6 mice or *Mlk3*−/− mice and tested for fMLP-stimulation in the Motility assay. fMLP-induced motility of neutrophils from C57Bl/6 mice was stimulated over six-fold ($p < 0.01$) while no increase in motility was observed in neutrophils from *Mlk3*−/− mice (Fig. 4C).

3.6 Pharmacological or genetic blockade of MLK3 activity impairs F-actin formation in fMLP-stimulated neutrophils

FMLP initiates motility in neutrophils and reduction of MLK3 activity reduces that motility. One possible cause of reduced motility is a limitation of the cell's ability to form F-actin. To test this hypothesis, neutrophils from wild-type C57Bl/6 and *Mlk3*−/− mice were exposed to 1 μM fMLP for two minutes, fixed, permeabilized and stained with F-actin-specific FITC-phalloidin. Neutrophils that formed F-actin had a distinct extranuclear ring of intense fluorescence; those that had not formed F-actin appear weakly stained. Examples of F-actin positive (arrows) and negative cells are presented in Fig. 5A.

The results are summarized in Fig. 6B. The percentage of C57Bl/6 neutrophils with conspicuous F-actin increased from 25% to 65% following two-minute exposure to fMLP ($p < 0.001$). No such increase was observed in neutrophils from *Mlk3*−/− mice ($p = 0.364$). Similarly, pretreatment of C57Bl/6-derived neutrophils with URMC099 blocked the fMLP-induced increase in F-actin formation ($p < 0.05$).

3.7 MLK3 deficiency reduces fMLP-stimulated influx of circulating neutrophils into the peritoneum

We next tested whether the observed effects of MLK3 blockade on the *in vitro* migration of neutrophils could be recapitulated *in vivo*. To do this, we examined the effect of pharmacologic or genetic blockage of MLK3 on the influx of neutrophils from the

circulation into the peritoneum induced by fMLP. In C57Bl/6 mice pre-treated with 10 mg/kg URMC099 prior to stimulation by the intraperitoneal administration of 50 μg fMLP, we observed a significant reduction in the number of neutrophils that accumulated in the peritoneum after 30 minutes (Fig. 6). The complementary experiment in *Mlk3*−/− mice yielded a comparable result: the fMLP-stimulated accumulation of neutrophils 30 minutes following fMLP administration was not observed in *Mlk3*−/− mice and there was no incremental impact on neutrophil influx by URMC099 (Fig. 6). Taken together, these observations confirm the role of MLK3 in neutrophil migration *in vivo* and show that the inhibitory effect of URMC099 on neutrophil migration is mediated through its inhibition of MLK3 and not by effects on other kinases.

4. Discussion

The presence and activity of MLK3 has been studied in detail in neural cells and epithelia, but relatively little has been learned about the roles of MLK3 in cells of the lymphoid and myeloid lineages and particularly neutrophils. Here we have demonstrated that *Mlk3* is expressed in neutrophils. The expression of MLK3 in neutrophils has until now been assumed because multiple investigators have observed activation of the signaling molecules downstream from MLK3 including the MAP kinases p38 and ERK (Hakkim et al., 2011; Liu et al., 2012; Selvatici et al., 2006; Tsai et al., 2013; Zhong et al., 2003). We observed the expression of *Mlk3* mRNA by using primers that framed a segment of *Mlk3* sequence derived in part from exon 1 and in part from exon 2 to yield a characteristic 79-bp fragment. As expected, no *Mlk3*-specific PCR product was detected in neutrophils from the *Mlk3*−/− mice. This measurement of *Mlk3* gene expression by PCR was an indirect way to measure protein expression because available anti-MLK3 antibodies have not had the combination of affinity and specificity for the successful implementation of immunoprecipitation assays.

The presence of MLK3 was confirmed by the observed phenotypic equivalence of the response to the chemoattactant fMLP by neutrophils genetically lacking *Mlk3* and those treated with the MLK inhibitor URMC099 (Marker et al., 2013). In each of our tests, chemotaxis in a gradient of fMLP, movement stimulated by fMLP and F-actin formation stimulated by fMLP, URMC099 impaired fMLP-stimulation which demonstrates that the MLK3 protein was present in wild-type C57Bl/6 neutrophils and that it plays a role in governing cell motility.

The fMLP receptors (FPR) are GPCR which activate heterotrimeric G_i-proteins. In addition to other signaling molecules like PI3K and adenyl cyclase, the activated $G_i\alpha$ protein activates the rho-family GTPase cdc42 (Szczur et al., 2006) which in turn activates MLK3 by interaction with its CRIB domain, releasing MLK3's autoinhibition by its SH3 domain and promoting autophosphorylation of the catalytic domain and dimerization through the leucine zipper domain (Gallo and Johnson, 2002). Activated MLK3 then phosphorylates MKK3 which phosphorylates MAPK p38. Even though MLK3 has been implicated in the motility of multiple cell types (Chen and Gallo, 2012; Huang et al., 2004; Kovalenko et al., 2012; Mishra et al., 2010), the pathway connecting MLK3 and the cytoskeleton has not been elucidated.

There are at least two FPR in the mouse: the higher affinity FPR1 which has recently been identified as the source of internal signals that initiate neutrophil movement and the lower affinity FPR2 which acts as a source of stop signals presumably slowing cell movement when the cell arrives at a source of infection (Hartt et al., 1999; Liu et al., 2012). FPR1 activates MAPK p38 which prevents GRK2 inactivation of FPR1 while FPR2 activates Erk which activates GRK2 and promotes FPR1 internalization. Our data suggest, but do not directly prove, that fMLP-mediated activation of FRP1 results in activation of MLK3 in the

pathway to its activation of MAPK p38. Whether FPR2 also results in activation of MLK3 is uncertain, and merits further investigation.

Our demonstration that the blockade of MLK3 by either pharmacological or genetic means reduces, or at least delays, neutrophil migration from the circulation into the peritoneum is consistent with our observations of altered neutrophil motility *in vitro*. Further work will be required to discern whether this MLK3-dependent defect is neutrophil-specific or if it results from interference with MLK3 function in additional cell types that interact with neutrophils.

We note that previous studies using URMC099 showed that this small molecule had no effect on neutrophil influx into the brain, following intracerebral injection of the potent neuroinflammatory protein, HIV-1 Tat (Marker et al., 2013). This discrepancy could reflect differences in the underlying mechanisms of neutrophil recruitment by HIV-1 Tat versus fMLP, differences in the pathways that contribute to neutrophil recruitment into the peritoneum versus those that regulate neutrophil trafficking across the blood-brain barrier, or other factors.

The ability to pharmacologically modulate neutrophil mobility could have major clinical significance in conditions involving excessive or unregulated neutrophil accumulation such as sepsis, ischemia reperfusion injury, respiratory distress syndrome, and multi-organ failure as noted above. Here we have shown how MLK3 can be one potential target for therapeutic intervention. Because there multiple receptor-activating ligands that mobilize neutrophils by distinct receptors that activate overlapping pathways (Amulic et al., 2012), it is probable that the targeting of intermediates in signaling cascades will require multiple sites of intervention. For example, PI3K accelerates the chemotactic response to fMLP but is not required for it (Heit et al., 2008). Combinations of PI3K inhibitors (Banham-Hall et al., 2012) and MLK3 inhibitors may be sufficient to modulate neutrophil migration and reduce damage at sites of inflammation (Bullone et al., 2013; Coffer et al., 1998).

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ABBREVIATIONS

AKT	Protein Kinase B
AD	Accumulated Distance
APC	allophycocyanin
CRIB	cdc42/Rac-interactive binding
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminotetraacetic acid
ERK	Extracellular Signal-Related Kinase
EtBr	ethidium bromide
FITC	fluorescein isothiocyanate
fMLP	N-Formyl-L-methionyl-L-leucyl-L-phenylalanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GPCR	G-Protein Coupled Receptor
GTP	guanosine triphosphate
HBSS	Hank's Buffered Salt Solution
ICAM	Intercellular Adhesion Molecule
JIP	JNK Interacting Protein
JNK	c-jun N-terminal Protein Kinase
MKK	Mitogen-activated protein Kinase Kinase
MLK3	Mixed Lineage Kinase 3
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PE	phycoerythrin
PEG	polyethyleneglycol
PI3K	Phosphoinositide-3-kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SH3	Src homology domain 3
TBS	Tris-buffered saline
TNF	Tumor Necrosis Factor

References

Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 2012; 30:459–89. [PubMed: 22224774]

Banham-Hall E, Clatworthy MR, Okkenhaug K. The Therapeutic Potential for PI3K Inhibitors in Autoimmune Rheumatic Diseases. Open Rheumatol J. 2012; 6:245–58. [PubMed: 23028409]

Barthwal MK, Sathyanarayana P, Kundu CN, Rana B, Pradeep A, Sharma C, Woodgett JR, Rana A. Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. J Biol Chem. 2003; 278:3897–902. [PubMed: 12458207]

Brancho D, Ventura JJ, Jaeschke A, Doran B, Flavell RA, Davis RJ. Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades. Mol Cell Biol. 2005; 25:3670–81. [PubMed: 15831472]

Bullone M, Moran K, Lavoie-Lamoureux A, Lavoie JP. PI3K and MAPKs regulate neutrophil migration toward the airways in heaves. J Vet Intern Med. 2013; 27:164–70. [PubMed: 23194017]

Chen J, Gallo KA. MLK3 regulates paxillin phosphorylation in chemokine-mediated breast cancer cell migration and invasion to drive metastasis. Cancer Res. 2012; 72:4130–40. [PubMed: 22700880]

Coffer PJ, Geijsen N, M'Rabet L, Schweizer RC, Maikoe T, Raaijmakers JA, Lammers JW, Koenderman L. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. Biochem J. 1998; 329(Pt 1):121–30. [PubMed: 9405284]

Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell. 1994; 77:841–52. [PubMed: 7911739]

Eash KJ, Means JM, White DW, Link DC. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. Blood. 2009; 113:4711–9. [PubMed: 19264920]

Elphick GF, Sarangi PP, Hyun YM, Hollenbaugh JA, Ayala A, Biffl WL, Chung HL, Rezaie AR, McGrath JL, Topham DJ, Reichner JS, Kim M. Recombinant human activated protein C inhibits integrin-mediated neutrophil migration. Blood. 2009; 113:4078–85. [PubMed: 19244161]

Flurkey, K. The Jackson Laboratory Handbook on Genetically Standardized Mice. 6th Edition. The Jackson Laboratory; Bar Harbor, ME, USA: 2009.

Fortin CF, Mayer TZ, Cloutier A, McDonald PP. Translational control of human neutrophil responses by MNK1. J Leukoc Biol. 2013; 94:693–703. [PubMed: 23401599]

Gallo KA, Johnson GL. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol. 2002; 3:663–72. [PubMed: 12209126]

Goodfellow VS, Loweth CJ, Ravula SB, Wiemann T, Nguyen T, Xu Y, Todd DE, Sheppard D, Pollack S, Polesskaya O, Marker DF, Dewhurst S, Gelbard HA. Discovery, Synthesis, and Characterization of an Orally Bioavailable, Brain Penetrant Inhibitor of Mixed Lineage Kinase 3. J Med Chem. 2013a

Goodfellow VS, Loweth CJ, Ravula SB, Wiemann T, Nguyen T, Xu Y, Todd DE, Sheppard D, Pollack S, Polesskaya O, Marker DF, Dewhurst S, Gelbard HA. Discovery, Synthesis, and Characterization of an Orally Bioavailable, Brain Penetrant Inhibitor of Mixed Lineage Kinase 3. J Med Chem. 2013b Eprint ahead of pub.

Hakkim A, Fuchs TA, Martinez NE, Hess S, Prinz H, Zychlinsky A, Waldmann H. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. Nat Chem Biol. 2011; 7:75–7. [PubMed: 21170021]

Handley ME, Rasaiyaah J, Chain BM, Katz DR. Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity? Int J Exp Pathol. 2007; 88:111–26. [PubMed: 17408454]

Hartt JK, Barish G, Murphy PM, Gao JL. N-formylpeptides induce two distinct concentration optima for mouse neutrophil chemotaxis by differential interaction with two N-formylpeptide receptor (FPR) subtypes. Molecular characterization of FPR2, a second mouse neutrophil FPR. J Exp Med. 1999; 190:741–7. [PubMed: 10477558]

Heit B, Liu L, Colarusso P, Puri KD, Kubes P. PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP. J Cell Sci. 2008; 121:205–14. [PubMed: 18187452]

Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. J Cell Sci. 2004; 117:4619–28. [PubMed: 15371522]

Kim KY, Kim BC, Xu Z, Kim SJ. Mixed lineage kinase 3 (MLK3)-activated p38 MAP kinase mediates transforming growth factor-beta-induced apoptosis in hepatoma cells. J Biol Chem. 2004; 279:29478–84. [PubMed: 15069087]

Kovalenko PL, Kunovska L, Chen J, Gallo KA, Basson MD. Loss of MLK3 signaling impedes ulcer healing by modulating MAPK signaling in mouse intestinal mucosa. Am J Physiol Gastrointest Liver Physiol. 2012; 303:G951–60. [PubMed: 22917630]

Lecut C, Frederix K, Johnson DM, Deroanne C, Thiry M, Faccinetto C, Maree R, Evans RJ, Volders PG, Bours V, Oury C. P2X1 ion channels promote neutrophil chemotaxis through Rho kinase activation. J Immunol. 2009; 183:2801–9. [PubMed: 19635923]

Leung IW, Lassam N. Dimerization via tandem leucine zippers is essential for the activation of the mitogen-activated protein kinase kinase kinase, MLK-3. J Biol Chem. 1998; 273:32408–15. [PubMed: 9829970]

Liu X, Ma B, Malik AB, Tang H, Yang T, Sun B, Wang G, Minshall RD, Li Y, Zhao Y, Ye RD, Xu J. Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. Nat Immunol. 2012; 13:457–64. [PubMed: 22447027]

Mahmudi-Azer S, van Eeden SF. Neutrophil 'connectivity': key to neutrophil-mediated tissue injury? Crit Care. 2003; 7:285–7. [PubMed: 12930551]

Marker DF, Tremblay ME, Puccini JM, Barbieri J, Gantz Marker MA, Loweth CJ, Muly EC, Lu SM, Goodfellow VS, Dewhurst S, Gelbard HA. The new small-molecule mixed-lineage kinase 3 inhibitor URMC-099 is neuroprotective and anti-inflammatory in models of human immunodeficiency virus-associated neurocognitive disorders. J Neurosci. 2013; 33:9998–10010. [PubMed: 23761895]

Mishra P, Senthivinayagam S, Rangasamy V, Sondarva G, Rana B. Mixed lineage kinase-3/JNK1 axis promotes migration of human gastric cancer cells following gastrin stimulation. Mol Endocrinol. 2010; 24:598–607. [PubMed: 20150185]

Mota M, Reeder M, Chernoff J, Bazenet CE. Evidence for a role of mixed lineage kinases in neuronal apoptosis. J Neurosci. 2001; 21:4949–57. [PubMed: 11438570]

Nihalani D, Meyer D, Pajni S, Holzman LB. Mixed lineage kinase-dependent JNK activation is governed by interactions of scaffold protein JIP with MAPK module components. EMBO J. 2001; 20:3447–58. [PubMed: 11432832]

Olins DE, Olins AL. Granulocyte heterochromatin: defining the epigenome. BMC Cell Biol. 2005; 6:39. [PubMed: 16287503]

Rangasamy V, Mishra R, Sondarva G, Das S, Lee TH, Bakowska JC, Tzivion G, Malter JS, Rana B, Lu KP, Kanthasamy A, Rana A. Mixed-lineage kinase 3 phosphorylates prolyl-isomerase Pin1 to regulate its nuclear translocation and cellular function. Proc Natl Acad Sci U S A. 2012; 109:8149–54. [PubMed: 22566623]

Selvatici R, Falzarano S, Mollica A, Spisani S. Signal transduction pathways triggered by selective formylpeptide analogues in human neutrophils. Eur J Pharmacol. 2006; 534:1–11. [PubMed: 16516193]

Sukhumavasi W, Egan CE, Denkers EY. Mouse neutrophils require JNK2 MAPK for Toxoplasma gondii-induced IL-12p40 and CCL2/MCP-1 release. J Immunol. 2007; 179:3570–7. [PubMed: 17785791]

Swenson KI, Winkler KE, Means AR. A new identity for MLK3 as an NIMA-related, cell cycle-regulated kinase that is localized near centrosomes and influences microtubule organization. Mol Biol Cell. 2003; 14:156–72. [PubMed: 12529434]

Swenson-Fields KI, Sandquist JC, Rossol-Allison J, Blat IC, Wennerberg K, Burridge K, Means AR. MLK3 limits activated Galphaq signaling to Rho by binding to p63RhoGEF. Mol Cell. 2008; 32:43–56. [PubMed: 18851832]

Szczur K, Xu H, Atkinson S, Zheng Y, Filippi MD. Rho GTPase CDC42 regulates directionality and random movement via distinct MAPK pathways in neutrophils. Blood. 2006; 108:4205–13. [PubMed: 16931627]

Tsai YR, Wang YJ, Lee MR, Hsu MF, Wang JP. p38 Mitogen-activated protein kinase and extracellular signal-regulated kinase signaling pathways are not essential regulators of formyl peptide-stimulated p47(phox) activation in neutrophils. Eur J Pharmacol. 2013; 701:96–105. [PubMed: 23348708]

Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford). 2010; 49:1618–31. [PubMed: 20338884]

Xu Z, Maroney AC, Dobrzanski P, Kukekov NV, Greene LA. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. Mol Cell Biol. 2001; 21:4713–24. [PubMed: 11416147]

Zhang H, Wu W, Du Y, Santos SJ, Conrad SE, Watson JT, Grammatikakis N, Gallo KA. Hsp90/p50cdc37 is required for mixed-lineage kinase (MLK) 3 signaling. J Biol Chem. 2004; 279:19457–63. [PubMed: 15001580]

Zhang QG, Wang XT, Han D, Yin XH, Zhang GY, Xu TL. Akt inhibits MLK3/JNK3 signaling by inactivating Rac1: a protective mechanism against ischemic brain injury. J Neurochem. 2006; 98:1886–98. [PubMed: 16831194]

Zhong B, Jiang K, Gilvary DL, Epling-Burnette PK, Ritchey C, Liu J, Jackson RJ, Hong-Geller E, Wei S. Human neutrophils utilize a Rac/Cdc42-dependent MAPK pathway to direct intracellular granule mobilization toward ingested microbial pathogens. Blood. 2003; 101:3240–8. [PubMed: 12511425]

HIGHLIGHTS

- Mixed Lineage Kinase 3 (MLK3) is expressed in murine neutrophils.
- Inhibition of MLK3 by URMC099 reduces fMLP-induced neutrophil chemotaxis.
- URMC099 inhibits fMLP-induced cell motility, directionality and F-actin formation.
- URMC099 reduces the fMLP-induced migration of neutrophils *in vivo*.
- URMC099 treatment of neutrophils phenocopies genetic deletion of *Mlk3*.
- MLK3 may be a target for reducing excess neutrophil migration in human disease.

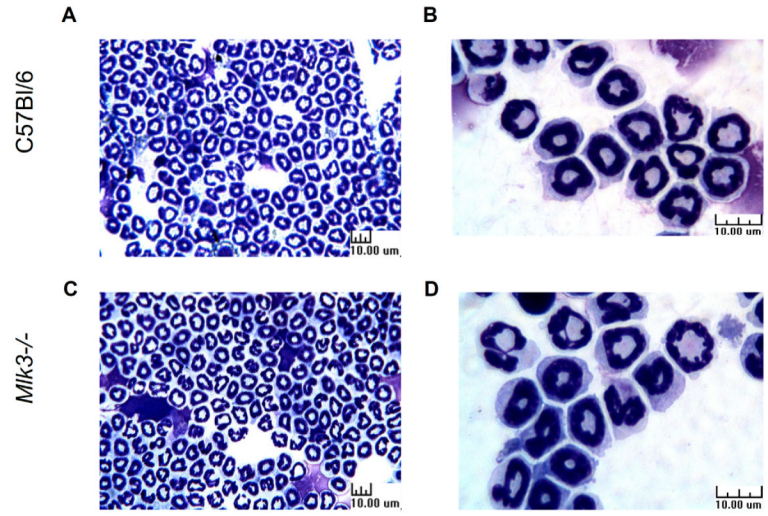

Figure 1. Neutrophils purified fromC57Bl/6 and *Mlk3*−/− mice

Neutrophils were isolated from the bone marrow of C57Bl/6 and *Mlk3*−/− mice using anti-Ly6G-conjugated magnetic microbeads, concentrated using a Cytospin, and stained with DiffQuick which contains xanthene and thiazine dyes. Images were taken using 40X (A and C) and 100X (B and D) objectives. Neutrophils from C57Bl/6 mice are in A and B; neutrophils from *Mlk3*−/− mice are in C and D. Scale bar is 10 μm.

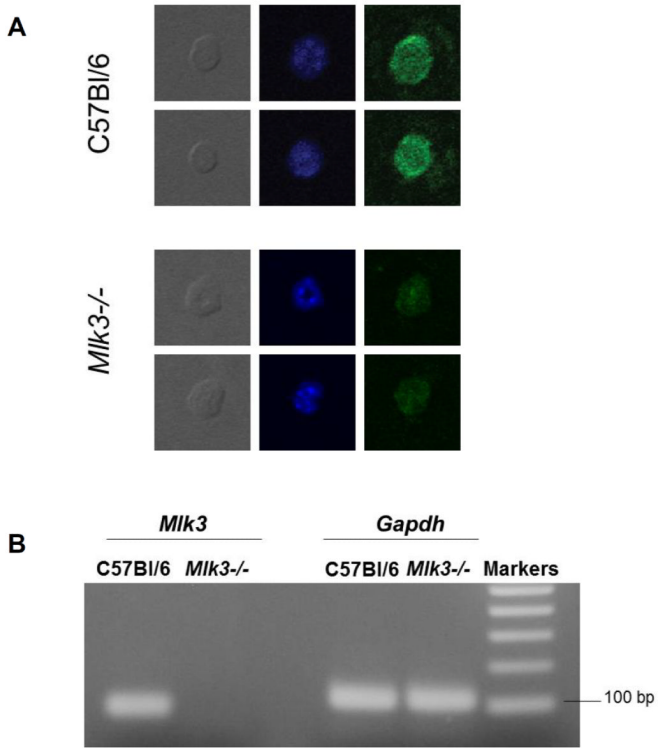

Figure 2. *Mlk3* is expressed in purified neutrophils from C57Bl/6 mice and not in neutrophils from *Mlk3*−/− mice

A) Neutrophils from C57Bl/6 (upper panel) and *Mlk3*−/− (lower panel) mice were purified and stained with Hoechst 33342 for DNA (blue) and anti-MLK3 and a fluorescent secondary antibody (green). Representative cells were then imaged by differential interference contrast microspcopy (left images), Hoechst 33342 fluorescence (center images) and anti-MLK3 fluorescence (right images). B) RT-PCR was used to detect *Mlk3* and *Gapdh* cDNA prepared from neutrophils isolated from the bone marrow of wild-type C57Bl/6 mice and knockout mutant *Mlk3*−/− mice. The presence and size of the resulting products were determined by electrophoresis in 2% agarose, staining with ethidium bromide and comparison against size standards; the 100 base pair standard is indicated. The characteristic product for *Mlk3* is 79 bp; that for *Gapdh* is 107 bp.

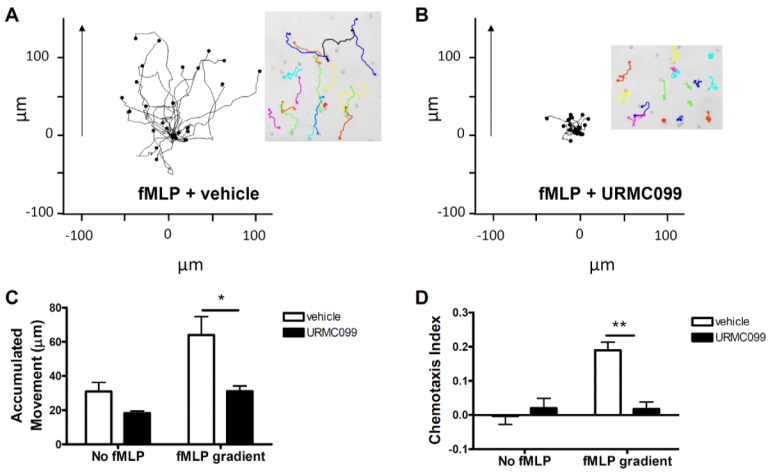

Figure 3. Movement of C57Bl/6 neutrophils in an fMLP chemotactic gradient in the absence and presence of the MLK3 inhibitor URMC099

Neutrophils isolated from C57Bl/6 mice were pre-treated with 100 nM URMC099 and then placed in a gradient of fMLP. Stimulated motility was measured by tracking the distance migrated by individual cells throughout a twenty-minute observation period. A,B) Representative tracings of the movement of individual neutrophils in a gradient of fMLP in the absence of URMC099 (A) and pre-treated with 100 nM URMC099 (B). Images were taken every minute for twenty minutes and analyzed using Image J software. The black arrows point towards the high concentration of fMLP to indicate the direction of the chemotactic gradient. C). Each bar is the average of 4–6 separate samples, > 30 individual neutrophils from each sample. Accumulated Distance is the average distance moved in μm by in the presence or absence of the fMLP gradient. URMC099 reduced the fMLP-stimulated movement of neutrophils to the levels observed in unstimulated neutrophils (63 μm compared to 30 μm); * p-value <0.05). D) Inhibition of MLK3 with URMC099 reduces chemoattractant-induced cell directional migration of neutrophils in a gradient of fMLP. Chemotactic Index is the ratio of the net movement in the direction of the fMLP and the Accumulated Distance, so it is dimensionless. URMC099 reduces the Chemotactic Index to the levels observed in cells in the absence of fMLP (0.18 compared to 0.04); **p-value < 0.01.

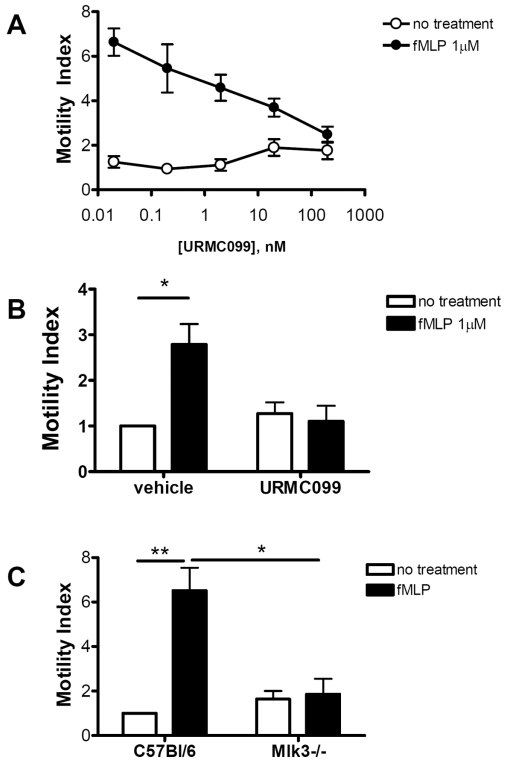

Figure 4. MLK3 inhibitor URMC099 reduces fMLP-stimulation of neutrophil migration in a dose-dependent manner

A) The motility index of fMLP-stimulated neutrophils decreases with increasing levels of URMC099; each data point represents the average of 20 cells. B) fMLP-stimulated migration following pre-treatment with medium containing vehicle (0.001% DMSO, vehicle) or 100 nM URMC099. * indicates p-value < 0.05. Each data point represents the average of 3 experiments, 20 cells per experiment.

C) The motility index was measured for neutrophils freshly isolated from C57Bl/6 mice and *Mlk3*- mice. Data represent the average of three separate experiments; 20 cells per experiment; ** p-value < 0.01. Error bars for the untreated C57Bl/6 condition (left bar) are absent because it is the reference against which the other conditions are normalized.

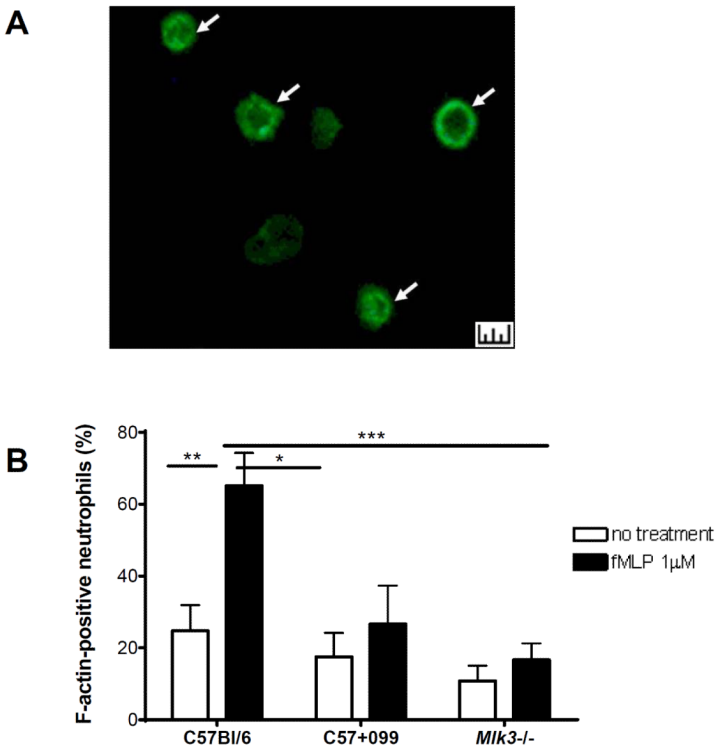

Figure 5. Pharmacologic or genetic blockade of MLK3 activity reduces F-actin formation in fMLP-stimulated neutrophils

A) Representative images of neutrophils stained with FITC-phalloidin to detect the formation of F-actin. Indicated by arrows, F-actin positive cells have extranuclear rings of intense fluorescence. F-actin negative cells appear weakly stained. B) The percentage of neutrophils that had formed F-actin rings 2 minutes following stimulation with 1 μM fMLP. Isolated C57Bl/6 neutrophils were pre-treated with vehicle (C57Bl/6), URMC099 (C57 + 099) prior to exposure to 1 μM fMLP. Neutrophils isolated from *Mlk3*−/− mice were treated only with vehicle prior to exposure to 1 μM fMLP. **$p < 0.01$.

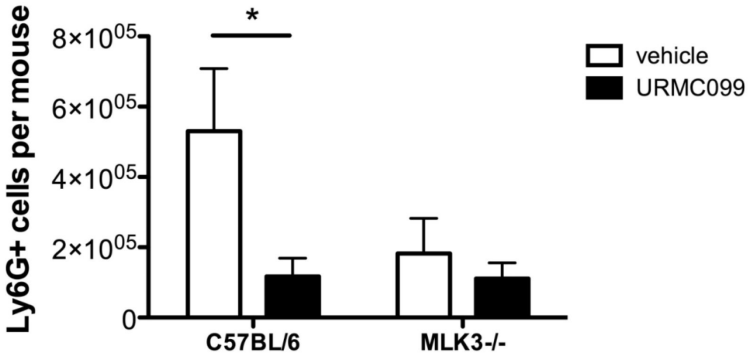

Figure 6. Pharmacologic or genetic blockade of MLK3 activity reduces fMLP-induced influx of neutrophils into the peritoneum

The total number of neutrophils (Ly-6G-positive) present in the peritoneal cavity 30 minutes after i.p. injection of 50 μg fMLP was determined by flow cytometry. Pre-treatment of C57Bl/6 mice with URMC099 yielded a significant reduction in the accumulation of Ly-6G-positive cells (left bars; *$p < 0.05$). *Mlk3*−/− mice also had a reduced accumulation of Ly-6G-positive cells compared to the C57Bl/6 mice but the reduction was not statistically significant ($p = 0.16$) and the incremental reduction in Ly-6G-positive cells in the *Mlk3*−/− mice resulting from URMC099 treatment was also not significant ($p = 0.57$).