Supporting Information

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SI Methods

Antibodies and Reagents. Monoclonal antibody against β-actin, FITC-conjugated Phalloidin, f-Met-Leu-Phe (fMLP), fibronectin, adenosine-5′-triphosphate (ATP) and L-α-phosphatidylinositol were purchased from Sigma. Monoclonal antibody against phosphatidylinositol 3,4,5-triphosphate was from Invitrogen. [γ-^{32}P]ATP was purchased from Perkin–Elmer. Monoclonal antibody against Rac1 and the transwell filters used for chemotaxis assays were obtained from BD Biosciences. Total Akt and phospho-specific anti-Akt^{T308} polyclonal antibodies were purchased from Cell Signaling Technologies. Polyclonal antibody against PI3Kγ was purchased from Santa Cruz Biotechnology. The horseradish peroxidase-conjugated secondary antibodies were obtained from Upstate Biotechnology. Chemiluminescence-based G-LISA kit to measure active RhoA was purchased from the Cytoskeleton Inc. The GST-PBD construct used for active-Rac1 binding was kindly provided by Dr. John O'Bryan of University of Illinois at Chicago.

Actin Polymerization Assay. Mouse BM neutrophils (1×10^7) in 200 μL of RPMI were left untreated or stimulated with 1 μM fMLP. At the given time point, cells were fixed by addition of 200 μL of cold 4% paraformaldehyde for 10 min. Cells were washed twice in PBS, permeabilized in 0.5% Triton X-100 for 10 min, and then incubated for 1 h at 4 °C with 0.2 μM FITC-conjugated Phalloidin. After staining, cells were washed twice in PBS and resuspended in 10% formalin. Samples were analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Immunofluorescence. Primary human neutrophils or mouse BM neutrophils in RPMI were left untreated or stimulated for 5 min with 100 nM fMLP. Cells were allowed to adhere to fibronectin-coated coverslips for 15 min at 37 °C, and then fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.5% Triton X-100 for 10 min and blocked overnight at 4 °C in PBS containing 0.1% Tween-20 and 2% FBS. Cells were stained for 1 h with primary antibody, washed three times, and incubated for 45 min with the appropriate fluorescently labeled secondary antibody. Following incubation, cells were extensively washed in PBS and coverslips were mounted with AntiFade reagent (Invitrogen). Samples were viewed on a Nikon Eclipse 300 fluorescence microscope under a 100× objective lens. Images were obtained using a cooled charge-coupled device camera and processed using MetaMorph software (Molecular Devices).

Lysate Preparation and Immunoblotting. Purified neutrophils (1×10^7/mL) in RPMI were left untreated or stimulated with fMLP for the appropriate time. Reactions were terminated and cells were lysed by the addition of equal volume of cold 2× lysis buffer [50 mM Tris, pH 7.4, 10% glycerol, 200 mM NaCl, 1% Triton X-100, 2 mM $MgCl_2$, and one tablet of Complete Protease Inhibitors (Roche)] and lysates were cleared by centrifugation. Aliquots of lysate were added to 2× Laemmli sample buffer and proteins were separated by 12% SDS/PAGE. Proteins were transferred to nitrocellulose membranes, blocked for 1 h in 5% milk and incubated overnight at 4 °C with the appropriate primary antibody. Membranes were washed and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5,000 dilution). The blots were visualized by enhanced chemiluminescence (ECL).

PI3Kγ Activity Assay. Mouse BM neutrophils, 2×10^7, were suspended in RPMI medium and prewarmed at 37 °C for 5 min. Cells were stimulated with 1 μM fMLP and the reaction was stopped by the addition of ice cold PBS. Cells were pelleted and lysed in 500 μL of lysis buffer on ice for 15 min. Cellular debris was centrifuged for 10 min at 12,000 × *g*. The supernatants were incubated with 4 μg of anti-p110γ (Santa Cruz Biotechnology) for 2h at 4 °C, followed by 1-h incubation with 50 μL of protein A-Sepharose beads. The beads were washed 3 times with lysis buffer and then 3 times with kinase buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, protease inhibitors). Kinase buffer, 50 μL, containing 20 μg of PI and 25 mM $MgCl_2$ were mixed with the beads for 10 min on ice. The reaction was initiated by the addition of 10 μL of 440 μM ATP containing 10 μCi of [γ-^{32}P]ATP and incubated for 20 min at 30 °C. The reaction was stopped by adding 20 μL of 1 N HCl, and lipids were extracted by addition of 200 μL of chloroform/methanol (1:1 vol/vol). Samples were centrifuged and the lower organic phase was applied to an oxalate-treated silica gel 60 plate. The plate was developed in 2-propanol-2 N acetic acid (2:1 vol/vol) and dried for 1 h. Radioactive lipid products were visualized by exposing the plate to X-ray film at −80 °C for 4 days.

Measurement of Total PI(3,4,5)P_3 in Neutrophils. Mouse BM neutrophils (1×10^7) in 200 μL of RPMI were either untreated or stimulated with 1 μM fMLP. At the given time point, cells were fixed by the addition of 200 μL of cold 4% paraformaldehyde for 10 min. Neutrophils were washed twice in PBS, permeabilized in 0.5% Triton X-100 for 10 min, and then incubated for 4 h at 4 °C with anti-PIP_3 monoclonal antibody. Cells were washed twice in PBS and incubated with TRITC-conjugated anti-mouse IgM for 1 h. Neutrophils were analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

Video microscopy. Mouse BM neutrophils in RPMI were plated on fibronectin and imaged after stimulation from a point source of 1 μM fMLP added via a micropipette (Femtotips). Neutrophils were viewed on a Nikon Eclipse microscope under a 20x objective lens.

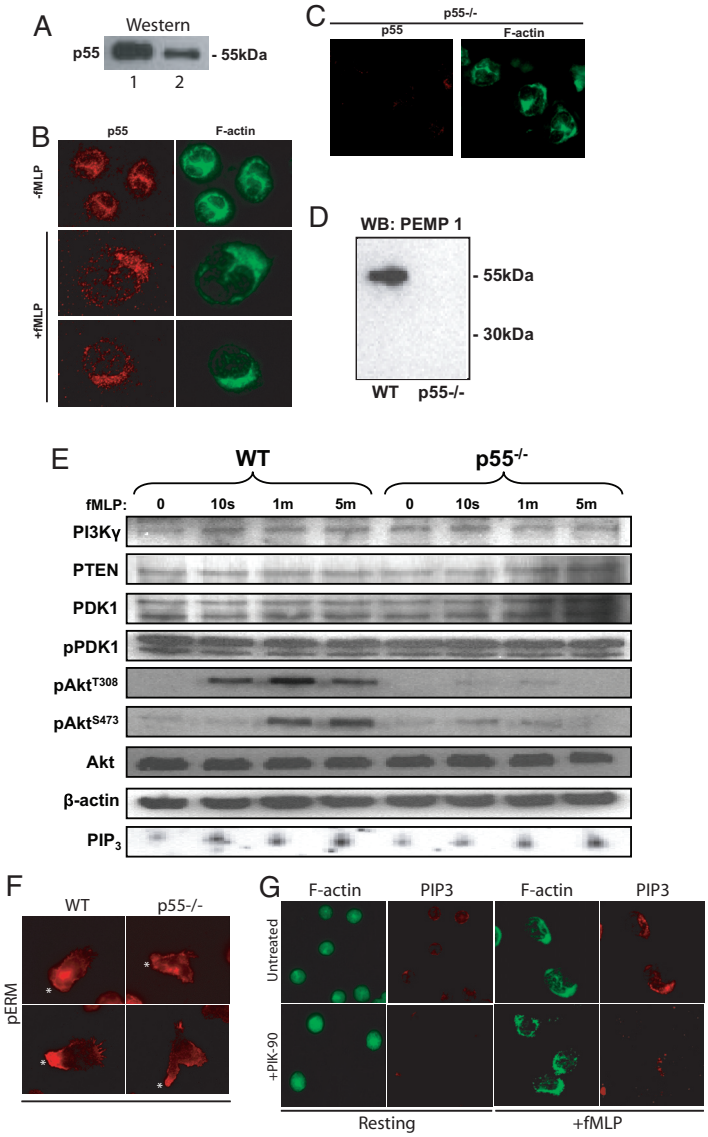
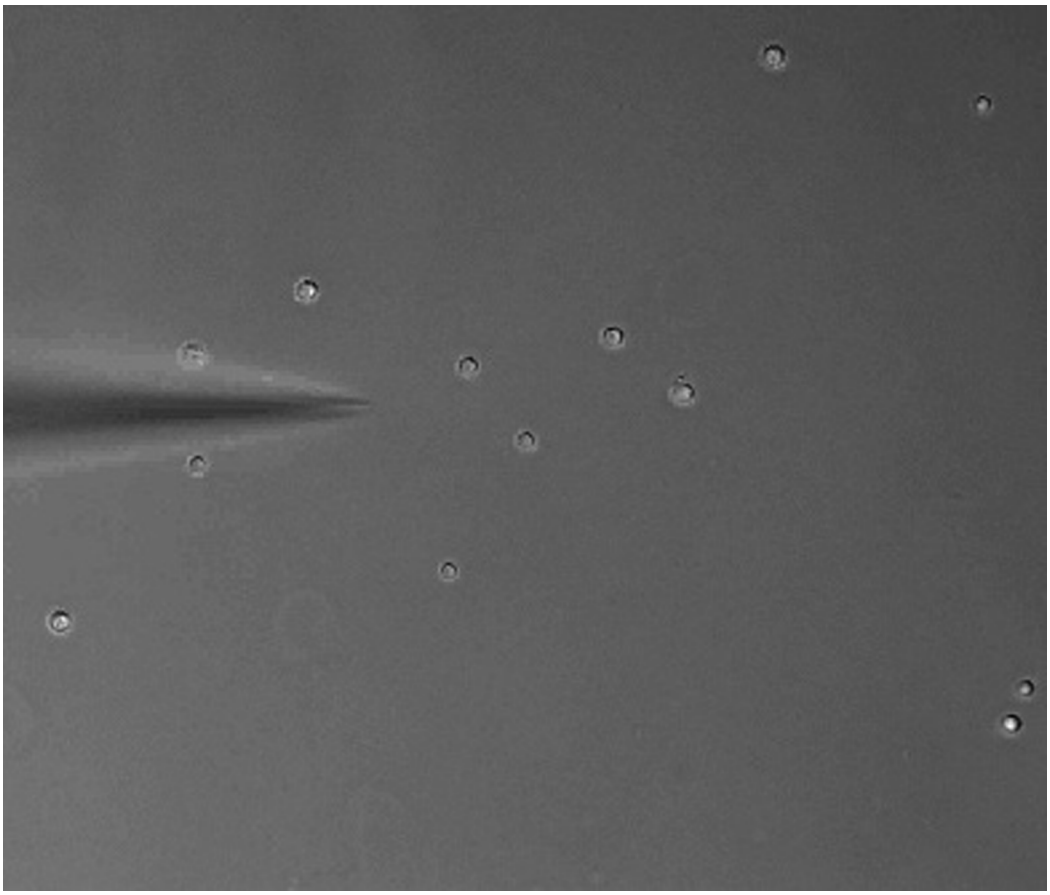
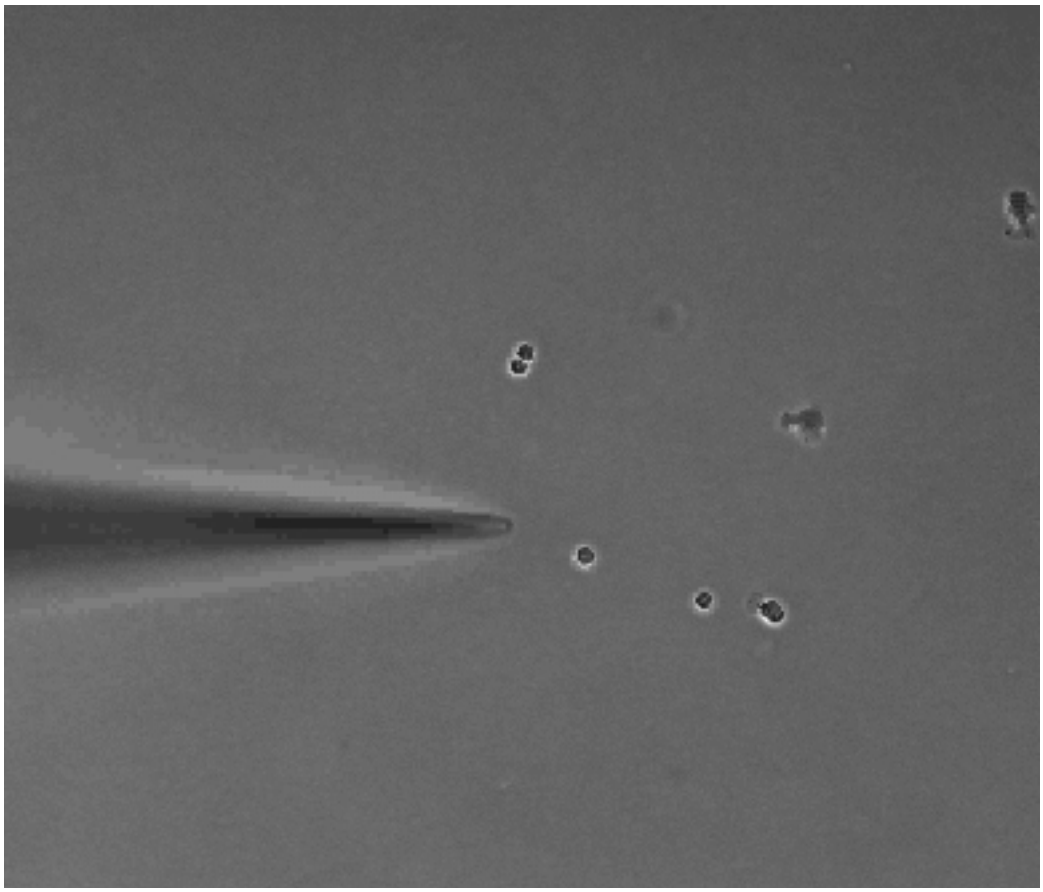


Fig. S1. Characterization of p55 in neutrophils. (*A*) Western blotting of p55. Fresh erythrocytes and neutrophils were isolated from the whole blood of healthy volunteers. The presence of p55 was confirmed in both erythrocyte ghosts (lane 1) and neutrophil lysate (lane 2) using a monoclonal antibody against p55. A single band of 55 kDa was detected in both samples. (*B*) Immunofluorescence analysis of p55 in human neutrophils. Purified neutrophils were plated onto fibronectin-coated coverslips, treated with 100 nM fMLP for 5 min, and stained for 1 h with p55 monoclonal antibody. For F-actin staining, cells were treated with FITC-conjugated Phalloidin. In resting neutrophils, p55 shows a diffuse staining pattern. Upon stimulation with fMLP, p55 translocated to the leading edge pseudopod marked by the F-actin staining. (*C*) Specificity of p55 monoclonal antibody. To confirm the specificity of the monoclonal antibody, $p55^{-/-}$ neutrophils were stained with p55 antibody and FITC-Phalloidin. No staining of p55 was detected in the $p55^{-/-}$ neutrophils. (*D*) To address the concern of leakiness from the N-terminal peptide, immunoblotting was performed with a polyclonal anti-peptide antibody termed PEMP 1. This antibody is specific for the 15-amino acid sequence (AA 65–80) located at the beginning of the N-terminal PDZ domain of human/mouse p55. No signal was detected in the $p55^{-/-}$ ghosts either at the full length 55 kDa or truncated N-terminal peptide at approximately 30 kDa, confirming the complete knockout status of $p55^{-/-}$ mouse model. (*E*) Western blotting of mouse BM neutrophils. Expression of various signaling proteins was analyzed by Western blotting of WT and $p55^{-/-}$ neutrophils. Proteins include PI3Kγ, PTEN, PDK1, phospho-PDK1, phospho-Akt^{T308}, phospho-Akt^{S473}, and Akt. β-actin was used as a loading control. Also included is the TLC for total PIP_3. In $p55^{-/-}$ neutrophils, a marked reduction in Akt activation was observed using antibodies against both phosphorylation sites. No other changes in protein expression were observed. (*F*) Phospho-ERM staining of WT and $p55^{-/-}$ neutrophils. pERM is a well-known uropod marker that localizes to the back of activated neutrophils. In $p55^{-/-}$ neutrophils, uropod-like structures are present, marked by the asterisks. (*G*) Sensitivity of the PIP_3 signal to a PI3Kγ inhibitor, PIK-90. Human neutrophils were treated with 1.0 μM of the PI3Kγ inhibitor, PIK-90, for 40 min before plating on fibronectin. Neutrophils were treated with or without 1.0 μM fMLP and stained for F-actin and PIP_3. In both resting and activated neutrophils, PIK-90 decreased the PIP_3 signal dramatically. Out of 200 neutrophils counted, less than 20% of the cells treated with PIK-90 show some PIP_3 immunofluorescence signal as compared to nearly 100% of untreated neutrophils positive for PIP_3 signal. These observations further validate the specificity of the PIP_3 monoclonal antibody.



Movie S1. Wild-type mouse BM neutrophils in RPMI were plated on fibronectin and imaged after stimulation from a point source of 1 μM fMLP added via a micropipette (Femtotips). Neutrophils were viewed on a Nikon Eclipse microscope under a 20$\times$ objective lens.

Movie S1 (AVI)



Movie S2. p55-null mouse BM neutrophils in RPMI were plated on fibronectin and imaged after stimulation from a point source of 1 μM fMLP added via a micropipette (Femtotips). Neutrophils were viewed on a Nikon Eclipse microscope under a 20$\times$ objective lens.

Movie S2 (AVI)