Supporting Information

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SI Methods

Harvesting of Murine Bone Marrow Cells and Differentiation into Bone Marrow-Derived Neutrophils. Bone marrow (BM) was flushed from WT mouse bones (femura, tibiae, and hips) in RPMI with L-glutamine, 1% penicillin/streptomycin, and 1% GlutaMAX using a 22-gauge needle (Becton Dickinson) in sterile conditions. The yield was $\sim 7 \times 10^7$ total BM cells per mouse. BM cells at 2×10^6 cells/mL per experimental condition were diluted in bone marrow-derived neutrophil (BMDN) media (α-MEM, 20% FCS, 1% penicillin/streptomycin, and 1% GlutaMAX) supplemented with the differentiating cytokines IL-3 (10 ng/mL final concentration) and granulocyte colony-stimulating factor (G-CSF; 20 ng/mL final concentration). Cells were cultured at 37 °C, 5% CO_2 for 3 d in T-75 flasks. On day 3, the media was replaced with fresh BMDN media (with 10% FCS) and supplemented with G-CSF only, and cells were cultured for three additional days. Amaxa nucleofection was used to transfect plasmid DNAs into macrophages (BMDM) or neutrophils (BMDN).

Gene Silencing. siRNA targeting PLD2 (exon 15; sense sequence GGA CUA CAG CAA UCU UAU CTT), PLD1 (exon 10; sense sequence GGC AAA UGA AGA GAU UUU UT), or siScrambled (a 19-bp scrambled sequence with 3′-dT overhangs certified to lack significant homology to any known gene sequences from mouse, rat, or human, and causes no significant changes in gene expression of transfected cells after 48 h at the same concentration as the siRNA in tests) were used to knock down gene expression using Mirus siQuest and Opti-MEM. siRNAs were then added to cells containing 1 mL Opti-MEM and incubated at 37 °C for 72–96 h to allow for maximum gene expression silencing, at which time cells were harvested and used for chemotaxis or PBD pull-down assays.

Baculovirus Cloning and Vector Construction. An upstream SalI restriction enzyme digestion (RED) site and a downstream HindIII RED site were introduced into the homologous fragments for each PCR product because of the sequences of the PCR primers. The PCR products for both PLD2 and Rac2 were amplified with pfu DNA polymerase using the primers published in ref. 1. The recombinant reaction between the linearized vector and the PCR product was performed with In-Fusion Dry-Down PCR Cloning Kit.

Sf21 Cell Culture. Sf21 insect cells were maintained at 2×10^6 cells/mL in Complete Grace's Insect Cell Culture Media containing 3.3 g/L yeastolate, 3.3 g/L lactalbumin hydrolysate, and 10% FCS at 30 °C in the absence of CO_2 using spinner flasks. For in vivo or in vitro assays, insect cells were maintained in monolayer cultures using 35-mm wells at 2×10^6 cells/well.

Generation of Purified, Recombinant Proteins from a Baculovirus/Insect Cell Expression System. A large-scale overexpression of both PLD2 and Rac2 was set up from baculovirus, starting from pBacC1 clones infected in Sf21 insect cells. We selected virulent Bac-C1-HA-Rac2, Bac-C1-myc-PLD2-WT, and Bac-C1-mycPLD2-ΔCRIB-1 recombinant viruses to infect Sf21 cells. Lysates from Sf21 cells (2×10^6 cells/mL) in a spinner of Complete Grace's Insect Cell Culture Media were used to bind 6xHN-tagged proteins in TALON resin (Clontech) according to the manufacturer's instructions. Washing buffer was 50 mM sodium phosphate (pH 7.0–7.5), 5 mM imidazole, and 300 mM NaCl; elution buffer was 50 mM sodium phosphate (pH 7.0–7.5), 500 mM imidazole, and 300 mM NaCl. OD at 280 nm was read from eluates of columns. Fractions were then dialyzed [5 mM Hepes (pH 7.8), 50 mM NaCl, 1 mM DTT, 5% glycerol] for 2 h. Aliquots were used for PAGE gels and for immunoblots that showed the prevalence of a protein of ∼22 kDa for Rac2 and, in separate experiments, of a protein of ∼110 kDa for PLD2.

Binding/Stoichiometry in an ELISA Plate Setting. To ascertain the stoichiometry of PLD2 and Rac2 binding, we used the recombinant proteins from a baculovirus/insect cell expression system. First, we added increasing concentrations of PLD2 to several wells in an ELISA plate (PVC grade, 96 wells) and determined the molar concentration of PLD2 bound by subtracting the number of moles in the supernatant (not bound) from the initial moles added to the wells. The number of moles in the supernatant was determined based on the absorbance at OD_{280}. After washing, blocking with 2% BSA, and 0.2% Tween-20, and further washing, we added a constant and excess amount of Rac2 to each condition of the PLD2 already bound to the ELISA plate. The plates were incubated at 30 °C for 2 h to allow proteins to interact. Last, we determined the molar concentration of Rac2 bound to the PLD2 (as moles added minus moles not bound in the supernatant) by direct UV absorbance at 280.

Rac2 PBD Pull-Down Assays. A total of 2×10^6 Sf21 insect cells were infected with Rac2 baculovirus at a multiplicity of infection (MOI) of 0.5:1 (number of virus particles:number of insect cells). PLD2-WT baculovirus was also coinfected using increasing MOIs from 5:1 to 50:1. Infections occurred for 48 h and were then activated using 3 nM EGF for 7 min at 37 °C. Cell lysates were prepared in lysis buffer (5 mM Hepes, pH 7.4, 0.1 mM sodium orthovanadate, 0.1% Triton ×-100) containing 0.5% digitonin. A total of 5 μL of PAK-1 PBD agarose was added to each sample and incubated at 4 °C for 30 min in the presence of magnesium lysis buffer. Samples were loaded onto gels, transferred to blotting membranes, and probed with α-HA antibodies to detect recombinant, GTP-bound Rac2, or α-myc antibodies to detect recombinant PLD2 that interacted with Rac2 in the pull-down assay. HRP-conjugated secondary antibodies were incubated with PVDFs and products visualized using ECL reagents. Where indicated, the Rac2 PBD pull-down assay was also conducted with purified, recombinant proteins instead of lysates.

GTP/GDP Exchange. A two-part methodology that involved, in separate experiments, [^{3}H]GDP dissociation and [^{35}S]GTP binding, is described below in detail. For these experiments, purified, recombinant proteins (PLD2 and Rac2) were used in an in vitro assay that had no phosphatydilcholine, the substrate of PLD2 action, so no enzymatic production of PA was possible. These reactions contained no PA unless expressly added exogenously, and, as such, indicated in *Results* and figure legends.

[^{3}H]GDP Dissociation. To measure the effect of PLD2 on [^{3}H]GDP dissociation from Rac2, 0.5 μg of Rac2 was preloaded with 2 μM [^{3}H]GDP. Simultaneously, 2 μg PLD2 was incubated for 10 min in 20 mM Tris·HCl (pH 7.5), 0.1 mM DTT, 80 mM NaCl, 0.5 mM $MgCl_2$, 0.8 mM AMP-PNP, and 1 mM GTP (80 μL vol). Preloaded [^{3}H]GDP-bound Rac2 samples were mixed with the buffer only or buffer containing PLD2 from the previous step (100 μL vol). Aliquots were taken at different times to measure the amount of radiolabeled [^{3}H]GDP bound to Rac2, spotted on Millipore BA85, air-dried, and washed 3× for 5 min with ice-cold 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$. The

amount of [^{3}H]GDP-bound Rac2 was measured by scintillation spectrometry.

[^{35}S]GTP binding. To examine if PLD2 could exchange GDP for [^{35}S]GTPγS on Rac2, 0.5 μg of Rac2 was incubated with 8 μM GDP, 6 mM $MgCl_2$ (20 μL vol) for 10 min at room temperature. GDP-bound Rac2 was added to 25 μL/100 μM AMP-PNP, 1 mM $MgCl_2$, and 1 μM [^{35}S]GTPγS ± 2 μg PLD2 (45 μL vol). The amount of [^{35}S]GTPγS-bound Rac2 was measured by scintillation spectrometry in terms of picomoles bound, as in ref. 2. For GDP dissociation and GTP binding (3), the Vav-1 (purified recombinant human Vav-1 GEF) was used as a positive control (OriGene Technologies).

PLD Activity Assay. Purified baculoviral PLD2 was processed for PLD activity in PC8 liposomes and [^{3}H]l-butanol beginning with the addition of the following reagents (final concentrations): 3.5 mM PC8 phospholipid, 45 mM Hepes (pH 7.8), and 1.0 μCi [^{3}H]l-butanol in a liposome form, as indicated in ref. 4, to accomplish the transphosphatidylation reaction of PLD. Samples were incubated for 20 min at 30 °C with continuous shaking. Addition of 0.3 mL ice-cold chloroform/methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by TLC. The amount of [^{3}H]PBut that comigrated with PBut standards (Rf = 0.45–0.50) was measured by scintillation spectrometry.

Cell Migration Assays. RAW264.7 macrophages were at 5×10^5 cells/mL in chemotaxis buffer (HBSS + 0.5% BSA). A total of 200 μL were placed in 8-mm pore, polycarbonate membrane Transwell inserts. Macrophage-CSF (M-CSF) was diluted to 3 nM in 500 μL chemotaxis buffer and placed into the lower wells of 24-well plates. Cell migration inserts were incubated for 1 h at 37 °C under a 5% CO_2 atmosphere.

Cell Polarization. Adherent cells were harvested by trypsinization, counted, and resuspended in chemotaxis buffer and 3 nM M-CSF. Cells were plated onto collagen-coated coverslips at a concentration of 5×10^4 cells/mL and incubated at 37 °C for specific times. The cell suspension was aspirated and adhered cells fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained with hematoxylin.

Phagocytosis. Zymosan A (*Saccharomyces cerevisiae*) bioparticles labeled with fluorescein isothiocyanate (FITC) were pre-incubated with IgG opsonizing reagent at 37 °C for 1 h. Following this, cells were incubated with opsonized zymosan (30 particles/cell). After gentle centrifugation of the plates to allow sedimentation of the beds, cells were allowed to undergo phagocytosis for 20 min. Manual counting was performed to calculate a phagocytic index.

Statistical Analysis. Data are presented as mean ± SEM. The difference between means was assessed by single-factor ANOVA. $P < 0.05$ indicated a significant difference.

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