Phosphatidic acid is a key lipid second messenger that mediates many crucial leukocyte-mediated cellular functions relevant to human health (embryological development, tissue repair, and immune surveillance/function) and pathology (vascular disease, chronic inflammatory diseases, and cancers), such as cell adhesion, migration, membrane remodeling, vesicular trafficking, and cell polarization (1). Defining and characterizing the molecular targets of PA in leukocyte functions is dependent upon PLD2, PA, and Arp3. A prolonged adhesion could effectively counteract the reversible intrinsic nature of cell adhesion and constitute a key player in chronic inflammation.

The Arp2/3 (actin-related proteins 2/3) complex is critical for the actin cytoskeleton to both control nucleation of actin polymerization and to form networks of branched actin in response to a variety of cellular stimulants (6). Two actin-related proteins, Arp2 and Arp3, and five additional subunits, ARPC1–5, form the Arp2/3 complex. Nucleation-promoting factors, such as the Wiskott-Aldrich syndrome protein (WASP) family (7), are responsible for signaling between Rho GTPase factors, such as Cdc42, Rho, and Rac and the Arp2/3 complex during actin polymerization (8). An activated WASP-Arp2/3 complex begins actin arborescent polymerization by creating multiple branch points that grow on the initial actin and form a functional actin cytoskeleton (9). This complex is involved in the establishment of cell polarity, found in macropinocytic cups in the leading edge of motile cells (lamellipodia) and in phagocytosis and wound healing (10).

Although Arp2/3 plays a central role in cell migration, the importance of Arp2/3-specific activation in the initial steps of leukocyte adhesion to solid surfaces (e.g. capillary beds) has not been examined to date. We present here a new concept in cell

2 The abbreviations used are: PLD, phospholipase D; WASp, Wiskott-Aldrich syndrome protein; PA, phosphatidic acid; PABD, PA binding domain; FIPI, N-[2-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl[ethyl]-5-fluoro-1H-indole-2-carboxamide; N-[2-(4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methyl[ethyl]-2-naphthalencarboxamide; NFOT, N-[2]-[3-dluoro-phenyl]-4-oxo-1,3,8-triazaspiro[4.5]dec-8-yl[ethyl]-2-naphthalencarboxamide; DOPA, 1,2-dioleoyl-sn-glycerol-3-phosphate; TRITC, tetramethylrhodamine isothiocyanate; DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine; ANOVA, analysis of variance; M-CSF, macrophage colony-stimulating factor.
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signaling with PLD and its enzymatic product, PA, that binds to the cellular motility machinery (chiefly Arp3) and leads to actin polymerization.

The two PLD mammalian isoforms (PLD1 and PLD2) play a concerted role in enhancing adhesion at differential temporal levels. This is important to establish a transient adhesion-depolymerization in the physiological context of inflammation. If this is deregulated, it could become a contributing cause of prolonged, chronic inflammation and its related diseases.

MATERIALS AND METHODS

Reagents—Low bicarbonate Dulbecco’s modified Eagle’s medium (DMEM) was from ATCC (Manassas, VA); Mirus Ingenio transfection reagent was from Mirus (Madison, WI); ECL reagent was from GE Healthcare; phalloidin-Alexa cells were immediately plated in 6-well plates

Fluor350 was from Invitrogen; DAPI, crystal violet, collagen, vitronectin, laminin, and fibronectin were from Sigma-Aldrich; macrophage colony-stimulating factor (M-CSF), MIP-1, MCP-1, and fetal calf serum were from Gemini-Bio Products (West Sacramento, CA); and [3H]butanol was from American Radiolabeled Chemicals (St. Louis, MO). Inhibitors used in this study were as follows: N-[2-[4-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]ethyl]-5-fluoro-1H-indole-2-carboxamide (FIPI) (Cayman Chemical, Ann Arbor, MI); N-[2-[4-(5-chloro-2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)-1-piperidinyl]-1-methylbutyl]-2-naphthalenecarboxamide (NCDOB) (Cayman Chemical); and N-[2-[1-(3-dluorophenyl)-4-oxo-1,3,–8-triazaspiro[4.5]dec-8-yl]-ethyl]-2-naphthalenecarboxamide (NFOT) (Tocris Biosciences, Bristol, UK). The plasmids used in the studies were as follows: pcDNA3.1-myPLD2-WT, pcDNA3.1-myPLD2-K758R, pCMV2-HAPLD1-WT, phCMV2-HAPLD1-K830R, pEGFP-N1-ACTR3 and Arp3-pmCherryC1 (both from Addgene.com), and pEGFP-Spo20PABD-WT.

GFP-based PA Sensor—Sporulation-specific protein 20 (Spo20) is a yeast protein required for the fusion of exocytic vesicles with the plasma membrane during yeast sporulation through its interactions with the SNARE complex (11–13), which contains an inhibitory region that sequesters the protein in the nucleus (13, 14) and a positive regulatory region that binds to phospholipids (especially PA) in the cell membrane, termed the PA binding domain (PABD). Cloning of the PABD into the pEGFP-1 vector, leading to pEGFP-Spo20PABD-WT, for use in microscopy of mammalian cells has been described (15, 16).

Cell Culture and Transfection of Macrophages—Murine RAW264.7/LR5 macrophages were from Dr. Dianne Cox (Albert Einstein School of Medicine Yeshiva University, New York) and were initially characterized by her (17) and were maintained in low bicarbonate Dulbecco’s modified Eagle’s medium in a humidified, 5% CO2 incubator. Viability assays were routinely conducted with 0.4% trypan blue stain in cell preparations prior to all analyses and were >95%. Cells were ultimately resuspended in DMEM, 0.1% BSA at a concentration 1.5 × 10^6 cells/ml for use in chemotaxis assays. A Murus-derived transfection protocol using the Ingenio reagent and electroporation was used to transfect plasmid DNAs into RAW264.7/LR5 mouse macrophages. When transfection was completed, cells were immediately plated in 6-well plates (non-tissue culture-treated plastic) in prewarmed RPMI-based medium supplemented with 20% FCS. Cells were cultured at 37 °C, 5% CO2 for 36 h to allow for maximum protein expression. Optimal protein expression was observed for 36–48 h post-transfection as verified using Western blot analyses of stimulated lysates.

Cell Adhesion—Cell adhesion experiments were performed on coverslips that were coated with collagen. For collagen coating, 10 µg/ml collagen in 20 mM acetic acid was prepared from the stock of 5 mg/ml collagen in 20 mM acetic acid. Coverslips were placed in 6-well plates, and 1 ml of collagen was added to each well and incubated at 37 °C for 1 h, after which collagen was aspirated, and the coverslips were gently rinsed with distilled water and air-dried. For the cell adhesion experiment, cells were harvested by trypsinization, counted, and resuspended in serum-free DMEM with 0.1% BSA and 3 mM MCP-1. Cells were plated on to the collagen-coated coverslips at a concentration of 5 × 10^4 cells/ml and incubated in 5% CO2 at 37 °C for specific time points. The cell suspension was aspirated, and 4% paraformaldehyde was added and incubated for 10 min at room temperature to fix the cells that were adhered. Cells were stained with hematoxylin.

Alternatively, instead of using a chemoattractant, such as MCP-1, for cell stimulation during adhesion assays, a phospholipid was used, specifically 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) from Avanti Polar Lipids (Alabaster, AL). It has been demonstrated that the mitogenic properties of PA are dependent on both its chain length and saturation (18). Long saturated fatty acid-containing PA is more mitogenic than PA with short saturated fatty acids (18). Similarly, the acyl composition of PA determines its binding abilities to the protein phosphatase 2C, with DOPA being more effective (19). DOPA was initially prepared in 0.5% fatty acid-free BSA in PBS at an initial concentration of 1 mM and sonicated to obtain unilamellar vesicles (20). BSA was used as the lipid carrier (21, 22). Ultimately, cells were stimulated with 0, 3, 10, 30, 100, 300, 1000, and 3000 nM concentrations of DOPA in serum-free DMEM with 0.1% BSA for ~15 min during adhesion for dose-dependent adhesion assays or with 300 nM DOPA in serum-free DMEM with 0.1% BSA for time-dependent adhesion assays.

PLD Activity Assay—Immunocomplex samples were processed for PLD2 activity in PC8 liposomes and n-[3H]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 n-[3H]butanol in a liposome form, as indicated (23). Samples were incubated for 20 min at 30 °C with continuous shaking. The addition of 0.3 ml of ice-cold chloroform/methanol (1:2) stopped the reactions. Lipids were then isolated and resolved by thin layer chromatography. The amount of [3H]But (phospho-butanol) that co-migrated with PBut standards was measured by scintillation spectrometry.

Detection of Polymerized Actin—RAW264.7 cells were transfected using plasmid DNA (if needed) and were plated onto coverslips placed into 35-mm tissue culture dishes. Eighteen h post-transfection, cells were fixed onto the coverslips using 4% paraformaldehyde for 10 min at room temperature. Cells were

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permeabilized using 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were blocked for 4 h at room temperature using 10% fetal calf serum in PBS and 0.1% Triton X-100 (PBS-T), washed three times with PBS-T for 5 min each, and then probed overnight at 4 °C in a 1:1000 dilution of rabbit α-Myc-TRITC antibody (specific for Arp3) (Santa Cruz Biotechnology, Inc.) (Fig. 6) in blocking buffer. Coverslips were again washed three times with PBS and then incubated in a 1:200 dilution of phallolidin-AlexaFluor350 in PBS for 2 h at room temperature. Slides were kept in the dark until needed and were viewed using a Nikon Upright Eclipse 50i tissue culture microscope; a Plan Fluor ×100/1.30 oil objective; and FITC, TRITC, or DAPI fluorescence filters. Photomicrographs were obtained using a Diagnostics Instruments Spot 6 digital camera and MetaVue software.

For imaging of polymerized actin in macrophages (Figs. 3 and 4), coverslips were incubated in a 1:200 dilution of phalloidin-TRITC in PBS for 2 h at room temperature, washed as previously stated, and then incubated in a 1:2000 dilution of DAPI in PBS for 5 min at room temperature.

For imaging of overexpressed PLD1 or PLD2 in macrophages (Fig. 5), coverslips were incubated in a 1:200 dilution of either rabbit anti-PLD1 or rabbit anti-PLD2 IgG antibodies (Santa Cruz Biotechnology) in blocking buffer for 4 h at room temperature, washed as described above, and then incubated with a 1:200 dilution of goat anti-rabbit TRITC IgG antibodies in blocking buffer for 1 h at room temperature, washed again as previously stated, and then incubated in a 1:2000 dilution of DAPI in PBS for 5 min at room temperature.

**Immunofluorescence Microscopy**—For detection of YFP-tagged PLD2 and polymerized actin (Fig. 3), we used an excitation filter of 490–510 nm, a dichroic mirror of 515 nm, and an emission filter of 520–550 nm (excitation/emission maxima of YFP are 512 and 529 nm, respectively). Actin was detected using phalloidin-AlexaFluor350 staining (as described above) viewed under a filter set with an excitation filter of 426–446 nm, a dichroic mirror of 455 nm, and an emission filter of 460–500 nm (excitation/emission maxima of CFP are 433 and 475 nm, respectively). In selected experiments, images of macrophages were acquired in a DeltaVision RT system (Applied Precision, Issaquah, WA) fitted with CFP and YFP filter sets for multichannel imaging and high resolution ×60 oil immersion objective.

**Immunoprecipitation, SDS-PAGE, and Western Blot Analyses**—After transfection, cells were harvested and lysed with special lysis buffer (5 mM HEPES, pH 7.8, 100 μM sodium orthovanadate, and 0.1% Triton X-100). The lysates were sonicated and the presence of overexpressed protein was confirmed by performing SDS-PAGE and Western blot analysis. For immunoprecipitation, the cell lysates were treated with 1 μl of monoclonal antibody specific for the respective protein of interest (α-HA IgG for HA-tagged PLD2 or α-actin IgG for actin) and 10 μl of agarose beads (Millipore, Billerica, MA) and incubated at 4 °C for 4 h. After incubation, the immunoprecipitates were washed with LiCl wash buffer (2.1% LiCl, 1.6% Tris-HCl, pH 7.4) and NaCl wash buffer (0.6% NaCl, 0.16% Tris-HCl, 0.03% EDTA, pH 7.4), respectively, and sedimented at 12,000 × g for 1 min. The resulting pellets were then analyzed using SDS-PAGE and Western blot analyses onto PVDF membranes that were probed for reactivity with either rabbit α-HA IgG for HA-tagged PLD2 or rabbit α-actin IgG for actin and subsequent goat anti-rabbit HRP IgG antibodies (all from Cell Signaling, Danvers, MA). Enhanced ECL reagents and autoradiography were used to detect immunoreactivities.

**Protein-Lipid Binding Assay**—The method for preparing and detecting protein-lipid binding has been described previously (24). Briefly, increasing concentrations of either DOPA or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids from Avanti Polar Lipids (Alabaster, AL) were spotted onto a PVDF membrane. DOPA or DOPC was dissolved in a 2:0.1:0.0.8 ratio solution of MeOH/CHCl3/H2O. Appropriate amounts of lipid ranging from 1 to 30 μg were spotted onto the membrane. The membrane was blocked overnight with a 3% fatty acid-free BSA solution. The membrane was then incubated overnight with recombinant Arp3 (50 nM). After protein incubation, the membrane was incubated overnight with a rabbit α-Arp3 IgG antibody. The membrane was then incubated with a goat anti-rabbit HRP IgG antibody, and the interaction was detected using chemiluminescence. Between incubation steps, the membrane was washed extensively with TBS-T.

**Actin Polymerization Assay**—A pyrene actin polymerization assay was used (25). RAW264.7/LR5 cells were either transfected with expression plasmids pcDNA-mycPLD2 or pcDNA-HAPLD1 for 2 days or treated with PA for 10 min prior to harvesting. The PA was prepared in 0.5% fatty acid-free BSA in PBS and sonicated to obtain unilamellar vesicles (20). BSA was used as the lipid carrier (21, 22). Cells were sonicated in actin lysis buffer (20 mM Tris-HCl, 20 mM NaCl, and 768 nM aprotinin). Ten μl of cell lysates were added to 85 μl of pyrene-labeled actin-containing buffer, which was purchased as a kit (BK003) from Cytoskeleton, Inc. (Denver, CO). Ten μl of actin polymerization buffer was added to the reaction for a final total volume of 105 μl. Actin polymerization was measured for 12 min at 30-s intervals at an excitation of 350–360 nm with a bandwidth of 20 nm and an emission of 401–411 nm with a bandwidth of 10 nm in a TECAN Safire2 at room temperature. An in vitro actin polymerization assay was performed as outlined in the manufacturer’s instructions (Cytoskeleton) except that the protein of interest (Arp3) was incubated with increasing concentrations of PA for 10 min prior to beginning the assay. The Arp3 recombinant protein was from Novus Biologicals (Littleton, CO).

**Statistical Analysis**—Data are presented as the mean ± S.E. The difference between means was assessed by the single factor analysis of variance (ANOVA) test. Probability of p < 0.05 was considered to indicate a significant difference.

**RESULTS**

**Adhesion Enhances the Kinetics of PLD1 and PLD2 Activities and Vice Versa**—M-CSF at 3 nm concentration was found to be an excellent agonist for the study of RAW264.7/LR5 macrophage cell adhesion to collagen-coated microscope coverslips when compared with non-stimulated control cells (Fig. 1A). Macrophages that were fixed to the collagen-coated coverslips and subsequently stained with crystal violet were visualized using bright field microscopy favored a bell-shaped curve dur-
ing adhesion that was both time- and agonist-dependent (Fig. 1, B and C). We found 30–60-min adhesion and 5 nM M-CSF to be optimal for chemoattractant-mediated adhesion of RAW264.7/LR5 macrophages alone in the absence of protein overexpression. Next, we measured the effect of agonist-mediated adhesion on the endogenous PLD activity of RAW264.7/LR5 macrophages and found that a variety of different macrophage agonists significantly increased PLD activity when compared with the mock-treated cells (Fig. 1D). Cells attached to several extracellular matrix proteins had increased PLD activity to varying degrees, with collagen being the highest (Fig. 1E). Because collagen coating yielded a greater increase in endogenous PLD activity of macrophages, we continued to use this matrix as our adhesion substrate in subsequent experiments.

Having observed that adhesion promoted an enhancement in PLD activity, we found that small molecule inhibitors specific for PLD1, PLD2, or both isoforms were effective in abrogating adhesion of RAW264.7/LR5 macrophages to collagen-coated surfaces (Fig. 2A). The dual PLD1/2 inhibitor FIPI, the PLD1-specific inhibitor NCDOB, and the PLD2-specific inhibitor NFOT reduced cell adhesion to collagen-coated substrates by 60–75% at 300 nM concentrations. Macrophages that overexpressed either PLD1 or PLD2 adhered more quickly to the collagen-coated substrate when compared with mock-transfected macrophages (Fig. 2B).

Further, when comparing both isoforms among themselves, PLD1 activity preceded that of PLD2. The kinetics experiments indicated that a rapid (t1/2 = 4 min) and transient activation of the PLD1 isoform occurred upon adhesion of macrophages, and a slower (t1/2 = 7.5 min) but more prolonged (>30 min) activation occurred for PLD2 (Fig. 2B). Cells that overexpressed mock, PLD1, or PLD2 were allowed to remain in suspension or adhere to microcentrifuge tubes for up to 30 min, at which point cells were harvested, and lysates were prepared for use in lipase assays.

As shown in Fig. 2C, overexpression of PLD2 increased ~2.5-fold the lipase activity from adhered cells when compared with PLD2-overexpressing suspension cells, which were similar in lipase activity to basal conditions, whereas overexpression of PLD1 did not yield a significant increase in PLD activity. Furthermore, lysates from macrophages that overexpressed either PLD1 or PLD2 and were immunoprecipitated using antibodies specific to either the Myc tag on PLD2 or the HA tag on PLD2 had significantly higher PLD activity when compared with the mock-transfected macrophages, and more importantly, PLD1 activity preceded that of PLD2 (Fig. 2D). These data indicate that cell adhesion to solid substrates is posi-
Effect of PLD Overexpression on Adhesion

Effect of Adhesion on PLD Activity

FIGURE 2. Effect of mammalian PLD isoforms (PLD1 and PLD2) on cell adhesion. A, functional effect of PLD inhibitors on cell adhesion. RAW264.7/LR5 macrophages were incubated in the presence of increasing concentrations of three different PLD-specific small molecule inhibitors for 20 min and were then used for adhesion experiments. B, effect of PLD overexpression on cell adhesion. Macrophages were mock-transfected or transfected with 2 μg of either a PLD1 or a PLD2 plasmid DNA. Forty-eight h post-transfection, cells were used for adhesion assays in the presence of 5 nM M-CSF for increasing periods of time. C, macrophages that overexpressed mock, PLD1, or PLD2 were used for PLD activity assay using cells that were allowed to remain in suspension or incubated in the presence of 5 nM M-CSF and allowed to adhere in microcentrifuge tubes for up to 30 min. Lysates were prepared and used for the PLD assay as detailed under “Methods and Materials.” D, macrophages that were allowed to adhere to collagen-coated surfaces were used to prepare cell lysates that were then immunoprecipitated using antibodies specific to either the HA tag on PLD1 or the Myc tag on PLD2. Immunoprecipitates were then used for the PLD activity assay. Experiments were performed in triplicate for at least three independent sets in total (n = 9). Results are mean ± S.E. (error bars) (calculated between independent experiments) and are expressed in terms of the number of cells adhered or, for PLD activity, in terms of dpm/mg of protein. *, statistically significant (p < 0.05) ANOVA increases between samples and controls.

Effectively affected by these phospholipases and that PLD overexpression increased adhesion, such that PLD and adhesion are interrelated.

PLD and Actin Interact during Adhesion and Enhance Actin Polymerization—Having observed that adhesion promoted enhanced PLD1 and PLD2 activities and vice versa (Figs. 1 and 2), we determined whether PLD activity affected adhesion dynamics via a direct interaction with actin, which is intrinsically linked to adhesion. Using macrophages that overexpressed either PLD1 or PLD2 that were then allowed to adhere to collagen-coated substrates in the presence of M-CSF, we observed numerous focal adhesion points around the cell membrane for both PLD1 and PLD2 that were enriched with polymerized actin (white arrowheads), as detected using a TRITC-labeled antibody specific for phalloidin in fixed cells (Fig. 3A).

We observed that this effect occurred as soon as cells were positioned over the solid substrate (<5 min). A similar effect was observed in macrophages that detected YFP-tagged PLD2 and phalloidin-AlexaFluor350-tagged actin in living cells (Fig. 3B). Additionally, we observed that PLD2 and actin form a protein-protein interaction using co-immunoprecipitation (Fig. 3C) and that increased PLD overexpression (either PLD1 or PLD2) also positively affected polymerization of actin using a pyrene-based assay (Fig. 3D).

In the case of overexpressed PLD and actin polymerization, we noticed that the slope in the linear portion of the graph that
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A  Mock  PLD1  PLD2
Phalloidin-TRITC
DAPI
Merged

B  Effect of PLD on Actin
YFP-PLD2  Phalloidin-AlexaFluor350  Merged

C  
| 110 kDa  | 50 kDa  |
|---|---|
| No M-CSF  | +5 nM M-CSF  |
| Mock  | HA-PLD2  | Mock  | HA-PLD2  | Mock  | HA-PLD2  |
| I.P. α-Actin, W.B. α-HA  | I.P. α-lG, W.B. α-HA  | I.P. α-Actin, W.B. α-Actin  | I.P. α-lG, W.B. α-Actin  |

D  POLYMERIZED ACTIN

Fluorescence units (RFU)

Time (min)

Slope PLD2: 1.01
Slope PLD1: 1.54
Slope Mock: 0.63
corresponded to early time points (<10 min) increased in the following manner: PLD2 > PLD1 > mock (Fig. 3D). Collectively, data presented in Fig. 3 indicate a robust effect of PLD overexpression on the kinetics of actin polymerization because this parameter is key to the establishment of adhesions to surfaces.

PLD-produced PA Is a Requirement for Macrophage Adhesion, Which Changes the Kinetics of Actin Nucleation—Next, we determined whether the presence of the product of the PLD reaction, PA, was sufficient to mediate cell adhesion. In macrophages that overexpressed the lipase-dead PLD1 mutant (K866R) or lipase-dead PLD2 mutant (PLD2-K758R), both incapable of producing PA, adhesion was significantly decreased compared with that of macrophages that overexpressed the lipase-active PLD1-WT or lipase-active PLD2-WT (Fig. 4A). Adhesion of macrophages that overexpressed wild-type PLD1 or PLD2 increased to ~2-fold greater than that of the overexpressed lipase-dead mutant PLDs. Further, macrophage adhesion increased notably in the presence of an increasing concentration of PA with a maximal level of adhesion achieved using ~100–300 nM PA (Fig. 4B), and the kinetics of adhesion was substantially changed by the presence of PA (compare t50 of 3.2 min versus 2.5 min in the presence of PA) (Fig. 4C). All of these data suggest that the enzymatic activity of PLD (i.e., the generation of PA) is necessary for macrophage adhesion.

PA Mediates Actin-based Adhesion of Several Immune Cells to Solid Surfaces—We next wanted to know whether the PLD-derived PA enhancement of cell adhesion in macrophages would also extend to other innate immune cell types. First, we found that PA had a positive effect on human neutrophil adhesion and show in Fig. 4D that human neutrophils that were treated with PA had increased ruffle formation (right panel, white arrowheads) compared with non-treated control neutrophils (left panel). These panels suggest that adhesion of human neutrophils to a collagen-coated substrate was mediated by polymerized actin and PA when compared with PC-treated or control samples. Second, we found that PA had a positive effect on another macrophage cell line, differentiated THP-1 (Fig. 4, E–G), whereby the maximal effect of increasing PA concentration on cell adhesion/contact focal formation of dTHP-1 cells occurred at ~100 nM PA for ~10 min (Fig. 4, F and G, respectively).

Last, preincubation of RAW 264.7/LR5 macrophages with exogenous PA for increasing lengths of time led to a significant increase in the slope of actin nucleation when cell lysates were assayed in vitro (Fig. 4, H and I). Data presented in Fig. 4 suggest the importance of the product of the PLD reaction, PA, to the formation of polymerized actin at the time of macrophage adhesion. Additionally, the presence of PA resulted in an increase in the formation of membrane ruffling and adhesion foci that are involved in cell adhesion to suitable surfaces.

**A Synergism between PA and Arp3 Increases Both Actin Polymerization and Adhesion**—It is known that to initiate actin polymerization, small GTPases and Arp2/3 are recruited to the lamellipodia, which enables chemotaxis (26). We wanted to know whether this was the case for the initial steps of cell adhesion to collagen-coated solid surfaces. As shown in Fig. 5, A and B, inhibiting either Rac2 or Arp3 using specific small molecule inhibitors (EHT58 or CK-548, respectively) decreased adhesion by ~70–80% (open circles), but this was reversed/partially prevented in the presence of PA (closed symbols) (note a 3–6-fold change in the IC50 concentrations of the inhibitors with PA). This might be due to the ability of PA to negate the effect of inhibitors (CK-548 inserts into the hydrophobic core of Arp2/3).

We then focused our efforts on Arp3 action/function. This was because the difference between Rac2 inhibition and Arp3 inhibition (in the presence of PA), showed that PA had more of an effect on Arp3.

Macrophones that overexpressed both Arp3 and PLD2 adhered to collagen-coated substrates to a greater extent than cells that overexpressed only PLD1, PLD2, or Arp3 alone compared with mock-treated controls (Fig. 5C). This positive effect on adhesion was also evidenced in a time-dependent manner in regard to Arp3 + PLD2 (Fig. 5D). Using immunofluorescence microscopy to detect PLD1 or PLD2 localization with Arp3 in macrophages, we detected that both Arp3 (green fluorescence) and PLD2 (red fluorescence) were both more intense in fluorescence at ~10–15 min of adhesion and were co-localized (yellow fluorescence) together outside of the nucleus to cytoplasmic regions in the macrophages (Fig. 5F), which we did not observe in macrophages that overexpressed both Arp3 and PLD1 (Fig. 5E).

**PA Binds Directly to Arp3**—Although we have shown thus far that lipase-active PLD enhanced macrophage adhesion and that the product of the PLD reaction, PA, also mediated increased adhesion to collagen-coated surfaces (Fig. 4) and increased actin polymerization (Fig. 3), we were not certain if PLD-derived PA directly controlled the positive effect of PLD2 on Arp3-mediated adhesion. We hypothesize that PA had to bind or alter a protein of the cell adhesion machinery. As explained in the Introduction, a chief protein would be the Arp2/3 complex. Therefore, we next determined to what extent

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**FIGURE 3.** PLD positively interacts with and affects actin. **A,** RAW264.7/LR5 macrophages transfected with 2 μg of PLD1 or PLD2 plasmid DNAs and 48 h post-transfection were adhered to collagen-coated coverslips in the presence of 5 nM M-CSF for 45 min. Specimens were then fluorescently labeled using a rabbit α-phallodin-TRITC IgG antibody and DAPI. Representative immunofluorescence microscopic images (n = 10) are presented showing the numerous points of macrophage attachment to the collagen-coated surface. **B,** macrophages were transfected with 2 μg of YFP-PLD2 plasmid DNAs, and 48 h post-transfection, they were adhered to collagen-coated coverslips in the presence of 5 nM M-CSF for 45 min. Endogenous polymerized actin was detected in these cells using phallolidin-AlexaFlour350 staining. Shown is z-stack of representative immunofluorescence microscopy (n = 8) of adherent macrophages that overexpressed YFP-PLD2 and phallolidin-AlexaFlour350-tagged actin. Actin-rich cell membrane adhesion contact points are shown with yellow arrowheads. **C,** RAW264.7/LR5 cells were mock-treated or transfected with 2 μg of HA-PLD2. Forty-eight h post-transfection, cells were incubated in the presence of 5 nM M-CSF, and lysates were prepared that were then immunoprecipitated (I.P.) with the indicated antibodies. Resulting Western blots of indicated antibodies. Results shown are representative of 4 independent experiments performed in triplicate. **D,** effect of PLD overexpression on actin polymerization from cell lysates. Macrophages were transfected, and lysates were prepared as in A and used for the pyrene-based in vitro actin polymerization assay. The slope for each line is shown. Results are represented as mean relative fluorescence units (RFU) ± S.E. (calculated between independent experiments) or three independent experiments performed in duplicate (n = 6).
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if any PA could interact with Arp3 and/or influence the action of Arp3.

First, in vitro actin polymerization was greatly enhanced by increasing concentrations of Arp3 protein and PA used in different combinations (Fig. 6, A and B) when compared with actin polymerization reactions that contained Arp3 alone or PA alone (Fig. 6, C and D). Reactions that contained both Arp3 and PA polymerized actin much quicker and reached higher maximal relative fluorescence values than the samples that lacked Arp3 and/or PA. We then tested whether PA would bind

FIGURE 4. Effect of PA on adhesion and actin nucleation. A, macrophage adhesion was performed in the presence of PLD-WT or the lipase-inactive mutant of PLD (PLD-KR). RAW264.7/LRS macrophages were untreated or transfected with 2 μg of PLD1 or PLD2 plasmid DNAs and, 48 h post-transfection, were adhered to collagen-coated coverslips in the presence of 5 nM M-CSF for 45 min. B–I, macrophages were used for either adhesion to collagen-coated surfaces or actin polymerization assays in the absence or presence of 300 nM PA as a function of time. B, dose response of PA on macrophage adhesion. C, macrophage adhesion kinetics in the absence or the presence of 300 nM PA. D–F, PA enhances adhesion of other immune cells. D, human neutrophils in suspension were incubated in the presence or absence of either PC or PA and then adhered to collagen-coated glass coverslips, fixed, and stained with α-phalloidin-FITC IgG antibodies. White arrows, membrane ruffling (representative of n = 8). E and F, adhered dTHP-1 cells were stained with α-phalloidin-TRITC to visualize polymerized actin and DAPI to visualize the nucleus. The effects of increasing PA concentration (F) or time (G) on cell adhesion/actin enrichment of dTHP-1 cells using collagen-coated coverslips are shown. D–G, immunofluorescence microscopy of n = 8 representative fields. H and I, effect of PA on actin polymerization. RAW 264.7/LRS macrophages were mock-treated or incubated in the presence of 300 nM PA for up to 15 min, and lysates were used for the measurement of actin polymerization in vitro. Experiments were performed in triplicate for at least three independent sets in total (n = 9). Results are mean ± S.E. (error bars) (calculated between independent experiments) and are expressed in terms of the number of cells adhered. * or #, statistically significant (p < 0.05) ANOVA increases or decreases, respectively, between samples and controls.
directly to Arp3 using a lipid-protein binding assay that measured Arp3 antibody immunoreactivity of Arp3 binding to PA that was prebound to a PVDF membrane and determined that this was indeed the case (Fig. 6E, top half of blot). As a negative control, PC was not able to bind to Arp3 (Fig. 6E, bottom half of blot). Regarding the region where PA can possibly interact with Arp3, it is known that proteins that bind to PA do not contain a well-defined recognition motif, and usually PA binds to the positively charged amino acid residues or surface-exposed hydrophobic residues or both (27–29).

Additionally, using immunofluorescence microscopy of macrophages that overexpressed a PA sensor (pEGFP-Spo20-
PABD-WT), which we have already successfully used and documented in the past (30, 31), and Arp3, we observed that both PA and Arp3 co-localized around polymerized actin (Fig. 6F). These data indicate that PA had a direct lipid-protein interaction with Arp3 that also localized to areas of polymerized actin in the adherent cell, and a synergism exists between the PLD catalytic reaction (PA) and Arp3 during actin polymerization, which is a necessary step for macrophage adhesion.

**DISCUSSION**

The original aim of this study was to discover new molecular targets for the lipid second messenger, PA, in cell signaling net-
works that could explain its effects in key cellular functions during leukocyte-mediated processes relevant to human health (embryological development, tissue repair, and immune surveillance/function) and pathology (vascular disease, chronic inflammatory diseases, and cancers), such as cell adhesion and migration (1).

We present here the key involvement of PLD in regulating adhesion that is mediated by the binding of its enzymatic product, PA, to Arp3 protein, which leads to actin polymerization. The two PLD mammalian isoforms (PLD1 and PLD2) played a concerted role in enhancing adhesion at differential temporal levels that coincided with the sequential activation of the phospholipases. PLD1 was involved in accelerating the early onset of nascent adhesion (such as the stages found in acute inflammatory diseases), whereas PLD2 was found to prolong or maintain continued adhesion (such as the stages found in mature adhesion during chronic inflammation).

Therefore, adhesion is a necessary first step for rolling leukocytes in blood to anchor to the capillary beds around an inflamed or injured tissue. This adhesion is strong but reversible as leukocytes migrate through the capillary endothelial cells and abandon the blood by the process of diapedesis. The presence of PLD1/2 timing found here could play a role in these steps.

Further, when leukocytes fight the invading pathogen, they must again adhere and mount an effective attack with lytic enzymes and oxygen radicals. If the leukocytes were to remain at the site of inflammation for prolonged periods of time, then chronic inflammation would ensue. Understanding the role of PLD, which we found contributes to adhesion, would allow us to gain insight into how to stop/reverse this type of detrimental cellular process and reduce the occurrence of such inflammatory diseases.

In Fig. 7, we propose a model that we believe explains cell contact and cell adhesion involving PLD, which is centered around a coordinated PLD/PA activity that receives cues from the solid substrate. The key events are as follows. (a) Adhesion of macrophages to collagen-covered plates activates both PLD1 and PLD2; in turn, these phospholipases regulate adhesion in a spatio-temporal way, leading to a prolonged and sustained cellular function. (b) Nascent adhesion (at ~5 min) and established (at ~15 min) adhesions were enhanced by PLD1 and PLD2, respectively. (c) Phosphatidic acid, a lipid second messenger, binds specifically to the Arp2/3 component Arp3. (d) PA-Arp3 binding led to activation of Arp and subsequent actin polymerization. PLD2 is activated at slightly later times, albeit in a prolonged manner that sustains adhesion and membrane ruffle formation. We conclude that the initial activation of PLD by collagen occurs as a result of nascent adhesion, and then a PA-Arp3-actin pathway increases focal adhesion in mature or established adhesions.
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(39). When PLD2 is enzymatically inactive, cell adhesion and migration are both inhibited with a concomitant decrease in cell elongation (40). PLD is important for leukocyte migration (41, 42).

In this study, PLD was found in fundamental ways to be implicated in cell adhesion. Our data have shown that the presence of PLD was conducive to both enhanced and prolonged activation of several motility molecules. The product of the PLD reaction, PA, was found to activate a component of the Arp2/3 complex to initiate actin nucleation, which is dependent on the presence of the PLD2 isoform. One component of the Arp2/3 complex bound to pre-existing actin filaments that were initially stimulated by PLD1-derived PA and formed a functional and dynamic actin-mediated attachment of cells to collagen-coated surfaces.

The spatial temporal interactions between PLD1 and PLD2, described here for the first time, enable a smooth transition from cell adhesion and tissues, establishment of cell polarity, and the initiation of chemotaxis during inflammation. PLD is a necessary target to be considered if one wants to clinically ameliorate chronic inflammation. In view of this report, PLD should now be considered a bona fide target to devise new pharmaceutical inhibitors to its enzymatic activity to avoid untoward effects of inflammation.

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REFERENCES

1. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R. (2003) Cell migration: integrating signals from front to back. Science 302, 1704–1709
2. Mahankali, M., Peng, H. J., Cox, D., and Gomez-Cambroner, J. (2011) The mechanism of cell membrane ruffling relies on a phospholipase D2 (PLD2), Grb2 and Rac2 association. Cell Signal. 23, 1291–1298
3. Chimini, G., and Chavrier, P. (2000) Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. Nat. Cell Biol. 2, E191–E196
4. Iyer, S. S., and Kusner, D. J. (2006) Assay of phospholipase D activity in cell-free systems. Methods Mol. Biol. 332, 281–298
5. Lehman, N., Di Fulvio, M., McCray, N., Campos, I., Tabatabaian, F., and Gomez-Cambroner, J. (2006) Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2. Blood 108, 3564–3572
6. Mullins, R. D., Heuser, J. A., and Pollard, T. D. (1998) The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc. Natl. Acad. Sci. U.S.A. 95, 6181–6186
7. Burian, L. E., and Soderling, S. H. (2013) Under lock and key: Spatiotemporal regulation of WASP family proteins coordinates separate dynamic cellular processes. Semin. Cell Dev. Biol. 24, 258–266
8. Hühnner, K., Schell, B., Aepfelbacher, M., and Linder, S. (2002) The acidic regions of WASp and N-WASP can synergize with CDC42Hs and Rac1 to induce filopodia and lamellipodia. FEBS Lett. 514, 168–174
9. LeClainche, C., Didry, D., Carlier, M. F., and Pantaloni, D. (2001) Activation of Arp2/3 complex by Wiskott-Aldrich Syndrome protein is linked to enhanced binding of ATP to Arp2. J. Biol. Chem. 276, 46689–46692
10. Magdalena, J., Millard, T. H., Etienne-Manneville, S., Launay, S., Warwick, H. K., and Machesy, L. M. (2003) Involvement of the Arp2/3 complex and Scar2 in Golgi polarity in scratch wound models. Mol. Biol. Cell 14, 670–684
11. Neiman, A. M. (1998) Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. J. Cell Biol. 140, 29–37
12. Neiman, A. M., Katz, L., and Brennewald, P. J. (2000) Identification of domains required for developmentally regulated SNARE function in Saccharomyces cerevisiae. Genetics 155, 1643–1655
13. Nakaniishi, H., de los Santos, P., and Neiman, A. M. (2004) Positive and negative regulation of a SNARE protein by control of intracellular localization. Mol. Biol. Cell 15, 1802–1815
14. Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997) A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. Proc. Natl. Acad. Sci. U.S.A. 94, 3046–3051
15. Zeniou-Meyer, M., Zabari, N., Ashery, U., Chasserot-Golaz, S., Haeberle, A. M., Demais, V., Bailly, Y., Gottfried, I., Nakaniishi, H., Neiman, A. M., Du, G., Frohman, M. A., Bader, M. F., and Vitale, N. (2007) Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. J. Biol. Chem. 282, 21746–21757
16. Su, W., Yeku, O., Olepu, S., Genna, A., Park, J. S., Ren, H., Du, G., Gelb, M. H., Morris, A. J., and Frohman, M. A. (2009) 5-Fluoro-2-indolyl deschlorohalopemide (FIPI), a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. Mol. Pharmacol. 75, 437–446
17. Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., and Greenberg, S. (1997) Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J. Exp. Med. 186, 1487–1494
18. Krakab, M. J., and Hui, S. W. (1991) The mitogenic activities of phosphatidate are acyl-chain-length dependent and calcium independent in C3H10T1/2 cells. Cell Regul. 2, 57–64
19. Zhang, W., Qin, C., Zhao, J., and Wang, X. (2004) Phospholipid D α 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc. Natl. Acad. Sci. U.S.A. 101, 9508–9513
20. Yoon, M. S., Sun, Y., Arauz, E., Jiang, Y., and Chen, J. (2011) Phosphatidate activates mammalian target of rapamycin complex 1 (mTORC1) kinase by displacing FK506 binding protein 38 (FKBP38) and exerting an allosteric effect. J. Biol. Chem. 286, 29568–29574
21. Venkataraman, K., Lee, Y. M., Michaud, J., Thangada, S., Ai, Y., Bonkovsky, H. L., Parikh, N. S., Habruckovitch, C., and Hla, T. (2008) Vascular endothelium is a contributor of plasma sphingosine 1-phosphate. Circ. Res. 102, 669–676
22. Scott, S. A., Xiang, Y., Mathews, T. P., Cho, H. P., Myers, D. S., Armstrong, M. D., Tallman, K. A., O’Reilly, M. C., Lindsley, C. W., and Brown, H. A. (2013) Regulation of phospholipase D activity and phosphatidic acid production after purinergic (P2Y6) receptor stimulation. J. Biol. Chem. 288, 20477–20487
23. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. Biochem. J. 345, 401–415
24. Dowler, S., Kular, G., and Alessi, D. R. (2002) Protein lipid overlay assay. Sci. STKE 2002, p68
25. Doolittle, L. K., Rosen, M. K., and Padrick, S. B. (2013) Measurement and analysis of in vitro actin polymerization. Methods Mol. Biol. 1046, 273–293
26. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell 97, 221–231
27. Stace, C. L., and Kitakasis, N. T. (2006) Phosphatidic acid and phosphatidylycerine-binding proteins. Biochim. Biophys. Acta 1761, 913–926
28. Itoh, T., Hasegawa, J., Tsuji, K., Kanaho, Y., and Takenawa, T. (2009) The tyrosine kinase Fer is a downstream target of the PLD-PA pathway that regulates cell migration. Sci. Signal. 2, ra52
29. Kooijman, E. E., and Burger, K. N. (2009) Biophysics and function of phosphatidic acid. Biochim. Biophys. Acta 1791, 881–888
30. Frondoza, K., Henkels, K. M., Frohman, M. A., and Gomez-Cambroner, J. (2010) Phosphatidic acid (PA) is a leukocyte chemotacticant that acts...
through S6 kinase signaling. J. Biol. Chem. 285, 15837–15847
31. Henkels, K. M., Boivin, G. P., Dudley, E. S., Berberich, S. J., and Gomez-Cambronero, J. (2013) Phospholipase D (PLD) drives cell invasion, tumor growth and metastasis in a human breast cancer xenograph model. Oncogene 32, 5551–5562
32. Du, G., Altschuller, Y. M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A. J., Bader, M. F., and Frohman, M. A. (2003) Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. J. Cell Biol. 162, 305–315
33. Ha, K. S., and Exton, J. H. (1993) Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. J. Cell Biol. 123, 1789–1796
34. Shin, I., Kweon, S. M., Lee, Z. W., Kim, S. I., Joe, C. O., Kim, J. H., Park, Y. M., and Ha, K. S. (1999) Lysophosphatidic acid increases intracellular H2O2 by phospholipase D and RhoA in rat-2 fibroblasts. Mol. Cells 9, 292–299
35. Jeon, H., Kwak, D., Noh, J., Lee, M. N., Lee, C. S., Suh, P. G., and Ryu, S. H. (2011) Phospholipase D2 induces stress fiber formation through mediating nucleotide exchange for RhoA. Cell. Signal. 23, 1320–1326
36. Zhang, Q., Lin, F., Mao, T., Nie, J., Yan, M., Yuan, M., and Zhang, W. (2012) Phosphatidic acid regulates microtubule organization by interacting with MAP65–1 in response to salt stress in Arabidopsis. Plant Cell 24, 4555–4576
37. Powner, D. J., Payne, R. M., Pettitt, T. R., Giudici, M. L., Irvine, R. F., and Wakelam, M. J. (2005) Phospholipase D2 stimulates integrin-mediated adhesion via phosphatidylinositol 4-phosphate 5-kinase Iyb. J. Cell Sci. 118, 2975–2986
38. Iyer, S. S., Agrawal, R. S., Thompson, C. R., Thompson, S., Barton, J. A., and Kusner, D. J. (2006) Phospholipase D1 regulates phagocyte adhesion. J. Immunol. 176, 3686–3696
39. Chae, Y. C., Kim, K. L., Ha, S. H., Kim, J., Suh, P. G., and Ryu, S. H. (2010) Protein kinase Cδ-mediated phosphorylation of phospholipase D controls integrin-mediated cell spreading. Mol. Cell Biol. 30, 5086–5098
40. Knoepp, S. M., Chahal, M. S., Xie, Y., Zhang, Z., Brauner, D. J., Hallman, M. A., Robinsson, S. A., Han, S., Imai, M., Tomlinson, S., and Meier, K. E. (2008) Effects of active and inactive phospholipase D2 on signal transduction, adhesion, migration, invasion, and metastasis in EL4 lymphoma cells. Mol. Pharmacol. 74, 574–584
41. O’Luanaiagh, N., Pardo, R., Fensome, A., Allen-Baume, V., Jones, D., Holt, M. R., and Cockcroft, S. (2002) Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. Mol. Biol. Cell 13, 3730–3746
42. Zouwail, S., Pettitt, T. R., Dove, S. K., Chibalina, M. V., Powner, D. J., Haynes, L., Wakelam, M. J., and Insall, R. H. (2005) Phospholipase D activity is essential for actin localization and actin-based motility in Dictyostelium. Biochem. J. 389, 207–214