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Phospholipase D1 Regulates Phagocyte Adhesion

Shankar S. Iyer,* Reitu S. Agrawal,* Christopher R. Thompson,* Steven Thompson,* James A. Barton,* and David J. Kusner2*†‡

Adhesion is a fundamental cellular response that is essential to the physiologic processes of development, differentiation, proliferation, and motility, as well as to the pathologic processes of inflammation, transformation, and metastasis (1–3). In the innate immune system, adhesion is critical to the mobilization of phagocytes to sites of infection and injury (4, 5). Adhesion of phagocytic leukocytes (neutrophils, monocytes, macrophages, and dendritic cells) is an important modulator of antimicrobial and cytotoxic functions, including the respiratory burst and secretion of microbicidal compounds and inflammatory mediators (6–11).

Multiple phospholipid-dependent signal transduction pathways contribute to the regulation and physiologic/pathologic consequences of adhesion (5, 12–14). Prominent among these are kinases of the type I PI3K and protein kinase C (PKC)3 families (5, 12, 14–16). The membrane phospholipid, phosphatidylinositol (PI) 4,5-bisphosphate (PI(4,5)P2), is a critical nexus in these adhesion-associated pathways, serving as the substrate for both PI3K (to generate PI(3,4,5)P3), as well as phospholipase C (PLC) (to generate the PKC activator, diacylglycerol (DAG)) (13). In addition, PI(4,5)P2 regulates actin polymerization through interaction with several actin-binding proteins, including gelsolin and profilin (15–18). Dynamic rearrangements of the actin cytoskeleton are required for adhesion and associated cellular functions, including motility (1–3, 13, 19). In phagocytes, the actin cytoskeleton plays an essential role in adhesion, phagocytosis, secretion, and the respiratory burst (20, 21).

These considerations on the involvement of phospholipid-mediated signaling pathways in the regulation of adhesion suggest a potential role for phospholipase D (PLD). PLD enzymes comprise a highly conserved, ubiquitous family of signaling components found in viruses, bacteria, and all eukaryotic organisms (22, 23). PLD catalyze the hydrolysis of membrane phospholipids, chiefly phosphatidylcholine, to yield the bioactive lipid, phosphatic acid (PA). The rationale for the involvement of PLD in adhesion includes: 1) PLD-induced production of PA stimulates PI(4,5)P2 synthesis via activation of PI 4-phosphate 5-kinase (PI4P5-K) (24–26). 2) The conversion of PLD-derived PA to DAG by PA phosphohydrolase (PAH) is a major source of PKC stimulation in many cell types (27–29). In fact, this coupled PLD-PAH pathway is the predominant route for sustained generation of DAG in neutrophils stimulated by chemotactic peptides or complement-opsonized zymosan (30–34). 3) PA stimulates the activity of PLD, providing amplification of this source of DAG (22). 4) PA itself directly stimulates specific isoforms of PKC (35–37). 5) PLD is physically and functionally linked to the actin cytoskeleton (38–45). Stimulation of PLD by physiologic and pharmacologic agonists results in its association with actin filaments (41). In vitro, PLD binds both monomeric G-actin as well as F-actin with polymerization-specific consequences for lipase activity (44–46). G-actin inhibits PLD activity, whereas F-actin enhances it. This coordinate regulation of PLD and actin has been demonstrated to be important to the process of phagocytosis, during which PLD1 and actin are both localized to the membrane of nascent phagosomes (41, 47).
The objective of this study was to test the hypotheses that: 1) PLD is activated during adhesion of phagocytic leukocytes, and 2) stimulation of PLD activity promotes cell adhesion.

Materials and Methods

Materials

Unless otherwise stated, materials were from previously published sources (41, 44, 48–51). Rabbit polyclonal anti-Ab to PLD1 was obtained from S. Bourgin (University of Laval, Quebec City, Quebec, Canada) (52). Additional anti-PLD1 Abs were obtained from Santa Cruz Biotechnology. mAb to paxillin was obtained from ZMed and anti-talin mAb was obtained from Sigma-Aldrich. mAb to CD18 (H52) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Alexa 488-phalloidin, Oregon Green- and Texas Red-Conjugated secondary Abs, Texas Red-conjugated zymosan, and latex beads (3-μm diameter) were obtained from Molecular Probes. Glass chamber slides were obtained from Fisher. PuGENE 6 and fibronectin were purchased from Roche. Collagen and fibrinogen were obtained from Sigma-Aldrich, collagen- and fibronectin-coated tissue culture plates were obtained from BD Biosciences, and calcine was obtained from Calbiochem.

Preparation of neutrophils and macrophage

Heparinized venous blood was drawn from healthy adult volunteers in accordance with a protocol approved by the Human Subjects Institutional Review Board of the University of Iowa. Polymorphonuclear leukocytes (PMN; neutrophils) were isolated by density gradient separation on Ficoll-Hypaque, dextran sedimentation, and hypotonic lysis of contaminating erythrocytes (53, 54). PBMC were isolated as previously described (48, 49) and cultured in Teflon wells for 5 days in RPMI 1640 (pH 7.4) with 20% fresh autologous serum, 1% penicillin/streptomycin, and 1% penicillin/streptomycin. Monocyte-derived macrophages (MDM) were purified by adherence to 6-well plastic tissue culture plates or glass chamber slides for 2 h at 37°C in 5% CO2. Monolayers were washed three times and then incubated in RPMI 1640 with 2.5% autologous serum, without antibiotics, for use in experiments. Effects of surface-coated macroparticles on viability of PMN and MDM were assessed by exclusion of trypan blue, and MDM monolayer density was determined by nuclei counting with naphthol blue-black stain (48, 49). Purity and viability of PMN and MDM preparations were >95% by Wright staining and trypan blue exclusion, respectively.

Cell lines

The human promyelocytic cell line, PLB, was obtained from American Type Culture Collection and cultured in RPMI 1640, 10% FBS, 1% penicillin/streptomycin (55). PLB cells were differentiated to a neutrophil-like phenotype by incubation in dimethylformamide (DMF) for 4 days (55, 56). The RAW 264.7 murine macrophage cell line was the gift of J. Engelhardt (University of Iowa) and was cultured in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin (57).

Transfection of RAW 264.7 cells with short-interfering RNA (siRNA)

A 21-mer oligonucleotide sequence corresponding to hPLD1 nucleotides 344–364 (AAGTTAAGGAAAAATTCAGGC) was selected as the target sequence (58) and the siRNA obtained from Dharmacon was selected as the annealed, purified, and desalted duplex. The sense oligonucleotide was labeled with Cy5 at the 5′ end. RAW 264.7 cells were transfected with siRNA to PLD1 or control “scrambled” siRNA (200 nM final concentration) in RPMI 1640 using PuGENE 6, as recommended by the manufacturer. After 8–10 h, the cells were replenished with serum containing complete medium and incubated for 3–5 days before use in experiments. Transfection efficiency was determined at 24 h by FACS; 100% of the cells were positive for Cy5, with an average 2.5-log increase in the level of Cy5 fluorescence.

PLD activity in intact cells

Primary human PMN or MDM were radiolabeled with [3H]lyso-phosphatidylcholine (50, 51). Radiolabeled cells were then washed three times and incubated in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin. After incubation with 10 μM [3H]lyso-phosphatidylcholine for 1 h, the cells were washed and resuspended in 5 ml of RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin at 106 cells/ml. One milliliter of the labeled cells was incubated with 0.5–1.0% ethanol for 2 min before stimulation to permit detection of the PLD-specific transphosphatidylidylation product, phosphatidylethanolamine (PEt). At the end of the experiment, lipids were extracted in 5 vol of chloroform: methanol (2:1, v/v) and separated by TLC in an ethylacetate:isoctane:acetic acid (9:5:2) solvent system (41, 50, 59). [3H]PEt and [3H]PEt were identified by comigration with pure standards, quantitated (in triplicate) by liquid scintillation spectrometry, and counts were normalized for the total amount of [3H]phospholipid in each experiment. All experiments with primary cells (PMN or MDM) used blood samples drawn from at least four different donors.

Adhesion assay

Adhesion of cells to plastic tissue culture plates was determined as previously described (60), with the following modifications. Human PMN (106 cells/ml) in HBSS +1% BSA were incubated with 2 μg/ml calcine-AM (4 mM stock solution in DMSO) for 30 min, washed, and resuspended in HBSS +1% BSA at 105 cells/ml. One milliliter of the PMN suspension was added to fibronectin (FN)-coated tissue culture plates for the indicated times, followed by washing and determination of the fluorescence of the adherent cells on a Gemini EM microplate spectrofluorometer (Molecular Devices). For determination of the effects of anti-CD18-blocking mAbs on PMN adhesion and PLD activity, PMN in HBSS +1% BSA were labeled with calcine-AM or [3H]lyso-PAF, as noted above. Cells were washed and resuspended in HBSS + at a concentration of 106 cells/ml and then incubated with anti-CD18-blocking mAbs, H-52 or M18/2 (1–10 μg/ml), or isotype-matched control, irrelevant mAb for 60 min at 37°C with gentle tumbling. Adhesion and PLD activity were determined as described above. In select experiments, the effect of exogenous di-octanoylphosphatidic acid (dC8-PA) on adhesion of RAW 264.7 macrophages was determined, as described previously (61), with minor modifications. dC8-PA was reconstituted by probe sonication to 1 mM in HBSS +, to which fatty acid-free BSA (1 mg/ml) was subsequently added. This solution, or its buffer control lacking dC8-PA, was added to cells for 10 min at 37°C, before the adhesion assay.

Confocal microscopy

Primary human MDMs or RAW 264.7 macrophages were adhered to glass chamber slides for 5–60 min at 37°C, followed by fixation in 3.75% paraformaldehyde for 15 min and permeabilization in ice-cold acetone (49, 62). Following incubation with blocking buffer (PBS, 5% BSA, 10% horse serum) for 1 h, polyclonal rabbit Ab to PLD1, murine mAb to paxillin, or Alexa-488-labeled phalloidin were added for 1 h. Samples were washed repeatedly and incubated with secondary anti-IgG Abs conjugated to Texas Red or Oregon Green for 1 h at 25°C. Following repeated washings, coverslips were mounted with buffered glycerol solution and nail polish. Confocal microscopy was performed on a Zeiss Laser Scan Inverted 510 microscope. At least 25 cells were imaged for each condition, and data are representative of at least four experiments with cells from different donors.

Determination of cellular F-actin content

The level of F-actin in suspension or newly adherent cells was determined by TRITC-phalloidin fluorescent staining assay, as previously described (63, 64). Primary human neutrophils or RAW 264.7 macrophages in HBSS + (106 cells/ml) were incubated in suspension with ethanol (1.0%) or buffer control, for 5 min at 37°C. In select experiments, RAW cells were incubated with siRNA to PLD1, scrambled siRNA, PuGENE6 alone, or buffer, as indicated above. Cells were added to plastic tissue culture wells for 5–30 min, and samples were washed to remove nonadherent cells. Adherent cells were fixed in 100 μl of 37% formaldehyde by incubation at 25°C for 15 min. F-actin was stained with 1 μM TRITC-phalloidin in HBSS containing 100 μM calcium and 10 μM magnesium. The cytoplasmic region of TRITC-phalloidin was prepared from the methanol stock by evaporation of solvent under N2 and resuspension by sonication in HBSS +. Unbound label was removed by washing three times with HBSS + and the bound label was extracted in methanol for 18 h. The relative fluorescence of the methanolic extracts was measured on a Gemini EM Spectramax microplate fluorescence spectrophotometer (Molecular Devices) using an excitation wavelength of 540 nm and an emission wavelength of 575 nm. To determine the F-actin content in suspension cells, aliquots of RAW 264.7 macrophages or PMN were sedimented in microfuge tubes and stained, as above.
Analysis of data

Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally distributed data were analyzed for statistical significance using the Student t test. Nonparametric evaluation of other data sets was performed with the Mann-Whitney U rank sum test (65).

Results

PLD activity is increased during adhesion of primary human neutrophils and differentiated myeloid cell lines

To evaluate the hypothesis that adhesion is associated with stimulation of PLD activity, the level of adhesion of primary human PMN to fibronectin-coated tissue culture wells was first determined using the fluorescent cellular label, calcine (60, 66). FN is a 440,000-kDa protein present in the extracellular matrix (ECM) and plasma which promotes the adhesion of multiple cell types, including PMN, under both physiologic and pathologic conditions (6, 7, 67, 68). PMN exhibited a time-dependent adhesion to FN, with a maximal adherence of 78 ± 5% at 1 h (Fig. 1A), consistent with previous studies (67, 68).

For the determination of PLD activity, PMN were radiolabeled with [3H]lyso-PAF and incubated in FN-coated wells or left in suspension. Resting PMN in suspension exhibited a very low basal level of PLD activity (Fig. 1, B and C) (69–71), which was similar to that reported in other quiescent primary cells, including macrophages (48, 72). Incubation of PMN in FN-coated tissue culture plates was accompanied by marked accumulation of PA, the product of PLD-mediated catalysis (Fig. 1B). This significant stimulation of PA levels was especially notable for its rapidity, reaching a maximal value of 9.1-fold (range, 8.4- to 9.8-fold), compared with PMN in suspension at 2–5 min (p < 0.001 for all comparisons between adherent and suspension cells). PA levels in adhering PMN remained elevated throughout the 60 min of the assay. The rate and time to maximal value of PA accumulation preceded the parallel kinetic parameters for adhesion of PMN, suggesting that generation of PA could contribute to the process of adhesion. Of note, incubation of suspended neutrophils with soluble FN resulted in no detectable increase in PA levels (data not shown).

Because PA lies at the nexus of multiple phospholipid biosynthetic and catabolic pathways, cellular levels of PA can be altered by several enzymes. However, in most cell types, including neutrophils, activation of PLD is the most common means by which rapid increases in PA are induced by physiologic stimuli (22, 23). To definitively determine whether this accumulation of PA resulted from stimulation of PLD activity, 0.5% ethanol was added to the incubation to serve as an alternative substrate for the PLD-specific transphosphatidylation reaction (22, 23). In the presence of ethanol, adhesion of PMN to FN-coated tissue culture plates was accompanied by significant accumulation of PEt, 4.5-fold (range, 4.2- to 4.8-fold) (p < 0.001 for each time point) compared with control PMN in suspension (Fig. 1C). This generation of PEt is specifically indicative of PLD activity (22, 23). As with PA generation, noted above, incubation of suspended neutrophils with soluble FN resulted in no detectable increase in PEt levels, compared with buffer-treated control cells (data not shown).

To further characterize the relationship between activation of PLD and adhesion, parallel experiments were performed with the human myeloid cell line, PLB, following its differentiation to a “neutrophil-like” phenotype by incubation in DMF for 4 days. DMF-differentiated PLB cells are a well-characterized model of PMN that have been extensively used for studies of
biochemical activation and functional responses (e.g., respiratory burst, granule exocytosis) (55, 56, 73, 74). Incubation of differentiated PLB cells in FN-coated wells was associated with rapid and sustained production of PA and PEt (Fig. 1, D and E), with kinetics that closely resembled that of primary PMN, and similarly preceded adhesion of the cells to the substrate (not shown). The magnitude of PA generation in PLB cells undergoing adhesion was 10.4-fold (range, 9.8–11.0-fold) greater than cells in suspension ($p < 0.001$ for each time point). Similarly, adherent cells generated 3.7-fold greater levels of PEt (range, 3.3–4.0-fold) than PLB cells suspended in buffer ($p < 0.01$ for each time point). Taken together, these data indicate that adhesion of human neutrophils and a human neutrophil-like cell line were associated with rapid and sustained stimulation of PLD activity.

**Inhibition of PLD activity is associated with reductions in PMN adhesion**

As a first step in assessing whether stimulation of PLD activity is causally related to PMN adhesion, we incubated cells in low concentrations of ethanol (0.25–1.0%), before adhesion to FN-coated wells. Ethanol inhibits PLD-dependent generation of PA by substituting for water as the nucleophilic acceptor of the phosphatidyl moiety (22, 75). In addition to the ability of this unique reaction mechanism to specifically identify PLD enzymatic activity, via generation of the corresponding phosphatidylalcohol, PEt (Fig. 1, C and E), this depletion of PA has been used to define a role for PLD in diverse physiologic processes, including several key antimicrobial mechanisms of neutrophils, monocytes, and macrophages (phagocytosis, respiratory burst, degranulation) (10, 34, 69, 71, 76–78).

Incubation of primary human PMN with ethanol resulted in concentration-dependent reductions in adhesion-associated PA generation, and corresponding increases in PLD-mediated production of PEt (Fig. 2A). In parallel with this depletion of PLD-mediated PA generation, ethanol produced dose-dependent decreases in the level of PMN adhesion to FN (Fig. 2B). The inhibition of PMN adhesion by ethanol could be detected as early as 2–5 min after addition of the cells to FN. Of note, the PLD-dependent generation of PEt is also detected at this very early time point (Fig. 1C). These concentrations of ethanol had no effect on PMN viability, assessed via exclusion of trypan blue (data not shown), in agreement with previous studies (10, 34, 54, 69, 71, 76–78). These data are consistent with the hypothesis that stimulation of PLD activity functions to promote neutrophil adhesion.

**Macrophage adhesion stimulates PLD activity in a $\beta_2$ integrin-dependent manner**

The diverse spectrum of phagocytic leukocytes (neutrophils, monocytes, macrophages, and dendritic cells) share many critical antimicrobial functions, including chemotaxis, adhesion, phagocytosis, reactive oxidant generation, and secretion of cytokines and cytotoxic compounds. The regulatory pathways that control these functions are comprised of both common and distinct components (5, 79–81). To test the hypothesis that activation of PLD is a common feature of phagocyte adhesion, we evaluated both primary macrophages as well as a macrophage cell line. Primary human MDM, radiolabeled in suspension with [3H]lyso-PAF, exhibited a 10.4-fold (range, 9.7–11.1-fold) increase in PLD activity (PA accumulation), upon adhesion to tissue culture plastic. The kinetics of adhesion-associated PLD activation in primary macrophages were similar to those of neutrophils and differentiated PLB cells, in that maximal PA levels occurred at 5 min, and PLD activity was sustained throughout the 60 min of the assay (data not shown).

To more comprehensively characterize adhesion-associated PLD activation in macrophages, we used the RAW 264.7 murine macrophage cell line, which has been extensively used as a model of macrophage physiology, cell biology, and biochemistry (79, 80). Adhesion of RAW 264.7 macrophages to diverse ECM proteins, including FN, fibrinogen, and collagen, as well as tissue culture plastic, was associated with marked stimulation of PLD activity (Fig. 3A). The magnitude of these adhesion-associated increases in PLD activity was underscored by the fact that they equaled or exceeded the level of PLD activation produced by stimulation with 100 nM PMA (787 ± 65 [3H]PA cpm at 60 min, mean ± SEM, $n = 3$). Similar to primary human neutrophils and differentiated PLB cells, this activation of PLD exhibited a rapid onset and was sustained throughout the 90-min duration of the assay. The rate of increase in PLD activity exceeded the rate of adhesion (data not shown), supporting the hypothesis that stimulation of PLD activity may regulate the process of adhesion. Incubation of macrophages with FN, fibrinogen, or collagen in solution, did not stimulate PLD activity (data not shown). These data support a model in which activation of PLD is a common feature of phagocyte adhesion to a broad range of physiologically relevant ECM proteins.

Integrins are heterodimeric surface proteins that play an essential role in cell adhesion to ECM proteins (1–3). The members of the leukocyte-specific $\beta_2$ integrin family (CD11a/CD18, CD11b/CD18, CD11c/CD18) are important mediators of phagocyte adhesion (2, 5). To test the hypothesis that $\beta_2$ integrins are involved in adhesion-associated increases in PLD activity, we used the anti-CD18 mAb, H52, that has previously been demonstrated to block the adhesion of phagocytic leukocytes (82, 83). Incubation of RAW 264.7 macrophages with H52 anti-CD18 mAb resulted in...
PLD1 colocalizes with actin filaments at the adhesive surface of primary macrophages

A potential mechanism by which PLD1 may regulate adhesion is via interactions with the actin cytoskeleton (38–40). We and others have demonstrated that PLD1 binds to actin in vitro (41, 43, 44, 46), with polymerization-specific consequences for lipase function. Monomeric G-actin inhibits PLD, whereas F-actin augments PLD activity (44). However, the mechanisms that regulate PLD-actin interactions in vivo are unknown.

As a first step in evaluating the hypothesis that PLD regulates adhesion via interactions with the actin cytoskeleton, we sought to determine whether PLD1 associates with actin filaments in adherent macrophages. Primary human monocyte-derived macrophages were adhered to glass coverslips, and the subcellular localization of PLD1 and actin filaments were determined by confocal microscopy. At the adherent interface, there was significant colocalization of PLD1 with actin filaments (Fig. 4, A and B). This colocalization was detectable at very early stages of adhesion (5 and 15 min) and progressed throughout the 120-min time period that was examined. In Fig. 4B, two-dimensional reconstructions of the indicated vertical and horizontal planes from the merged images are presented on the right and upper sides, respectively. These two-dimensional reconstructions demonstrate in greater detail that there are significant regions of colocalization of PLD1 and F-actin, as well as discrete foci of each protein that do not overlap. The colocalization of PLD1 and F-actin occurred in two morphologically distinct patterns: 1) associated with the plasma membrane, consistent with the cortical ring of actin filaments, and 2) as discrete, punctate foci located at the adhesion surface of the macrophage.

The spatial distribution of these latter punctate foci of PLD-actin colocalization at the adhesion interface suggested that these may represent focal contacts, the phagocytic adhesion structures that are homologous to the focal adhesions present in nonphagocytic adherent cells. To test this hypothesis, the distribution of the focal contact protein, paxillin, was determined. The paxillin staining was consistent with its distribution on focal contacts (Fig. 4C). PLD1 exhibited partial colocalization with paxillin, primarily in these focal contacts, and, to a lesser extent, at certain points along the cell cortex. In addition, there were areas in which PLD1 and paxillin exhibited distinct cellular localizations. For example,
PLD1 stains the cortical membrane much more strongly than paxillin. The higher magnification of the merged image with two-dimensional reconstructions (Fig. 4D) supports the hypothesis that PLD1 partially colocalizes with paxillin in focal contact regions. Taken together, these data support a model in which PLD1 exhibits significant colocalization with F-actin at the adhesion surface of macrophages, in both a cortical distribution and in focal contacts.

Inhibition of PLD activity decreases adhesion-associated actin polymerization

To determine the effects of adhesion-induced PLD activity on cellular F-actin content, we used a previously characterized, fluorescent TRITC-phalloidin assay (63, 64). Human neutrophils in suspension were treated with 1.0% ethanol or buffer control for 5 min, before adhesion to plastic tissue culture wells for 5, 15, or 30 min. Aliquots from both the suspension and adherent PMN (in the absence or presence of ethanol) were subjected to detergent extraction and their F-actin content was determined by direct staining with TRITC-phalloidin. Adhesion of control PMN to plastic was accompanied by rapid and sustained increases in F-actin content, in agreement with previous data (63) (Fig. 5). Inhibition of PLD activity with ethanol markedly reduced the adhesion-associated increase in PMN F-actin content (F-actin_{total} – F-actin_{Suspension}), decreasing it by 79–91% during the initial 30 min of adhesion, p < 0.003 for all time points examined (5, 15, and 30 min) (Fig. 5). These data support the hypothesis that adhesion-associated increases in PLD activity promote actin polymerization.

Specific reduction in macrophage PLD1 by RNA interference is associated with inhibition of adhesion

The two mammalian PLD isoforms, PLD1 and PLD2, share ~50% homology, but exhibit differences in mechanisms of activation and subcellular localization (22, 23). Human and murine phagocytes express both PLD1 and PLD2, and differences in their specific functions have begun to be elucidated (47, 84). PLD1 regulates macrophage phagocytosis and localizes to the forming phagocytic cup and to the nascent phagosome for approximately the first 30 min following ingestion (47, 85). Because the biochemical mechanisms that regulate phagocytosis and adhesion exhibit significant overlap (1, 3, 79, 80, 86), we hypothesized that PLD1 functions in macrophage adhesion.

RNA interference (RNAi) with purified duplex siRNA to PLD1 (58) was used to achieve a specific reduction of PLD1 in RAW 264.7 macrophages. Western blot confirmed PLD1 protein levels were decreased by ~65% (range, 59–71 reduction by densitometry, p < 0.01), compared with untreated cells, or those incubated with lipofection medium alone, or with scrambled control siRNA (Fig. 6A). Macrophages in which PLD1 was reduced by siRNA exhibited a 56–78% reduction in adhesion-associated PLD activity (p < 0.001), with the maximal effect detected at the earliest time point that was studied, 5 min (Fig. 6B). PLD1-depleted macrophages exhibited a parallel reduction in adhesion, which ranged from 52 to 73%, (p < 0.001) (Fig. 6C). These data support the hypotheses that PLD1 is activated during macrophage adhesion, and that this stimulation of PLD1 activity plays an important role in the process of adhesion.

To further evaluate this proposed role of PLD in phagocyte adhesion, we tested the effect of adding purified, cell-permeable PA (diC8-PA) to macrophages in which endogenous PLD1 had been reduced by RNAi. Aqueous-soluble lipids with acyl chains <8 carbons has been used previously to reconstitute lipid signal transduction, including that mediated by PLD (61, 87, 88). Incubation of PLD1-depleted RAW cells with diC8-PA (100 μM) reconstituted the level of adhesion to that of control cells treated with buffer alone, or scrambled siRNA (Fig. 6D). These data further support the role of PLD and its product, PA, in the process of adhesion.

Confocal microscopy of newly adherent (5–60 min) control RAW 264.7 macrophages (treated with scrambled siRNA) indicated a partial colocalization of PLD1 and F-actin that was similar to that exhibited by primary human macrophages. PLD1 was enriched at the adhesion interface and colocalized with actin filaments in the cell cortex and other F-actin-enriched
structures, including lamellipodia (Fig. 7A). The cortical colocalization of PLD1 and actin filaments was also present throughout the remainder of the cell, as demonstrated by z-slices taken at 3 μm above the adherent surface (Fig. 7B). In contrast, RAW 264.7 macrophages treated with siRNA to PLD1 exhibited irregular actin filaments at the adhesion interface, decreased lamellipodia, and reduced cortical actin throughout the cell (Fig. 7, C and D). For comparison with the control sample in Fig. 7, B and D, is the z-slice at 3 μm above the surface of a macrophage treated with PLD1-siRNA. Determination of cellular F-actin levels with the TRITC-phalloidin assay indicated that siRNA-mediated suppression of PLD1 was associated with significant reductions in F-actin content at the early stages of adhesion (Fig. 7E). At 5 min, the level of cellular F-actin was reduced by 61% (range, 59–63%; p < 0.01). These data support the hypothesis that PLD1 promotes actin polymerization and development of F-actin enriched structures in newly adherent macrophages. Reductions in the levels of PLD1 protein and activity via RNAi are associated with inhibition of adhesion, decreased levels of F-actin, and irregular actin filaments.

**Discussion**

The process of adhesion is fundamental to essential cellular functions, including motility, interactions with ECM, cell-cell contacts, and apoptosis. In the innate immune system, adhesion is central to the generation of inflammation, including extravasation of intravascular fluid into the extravascular space to produce edema, transmigration of phagocytes from the vasculature into tissues, and their chemotaxis to sites of infection and injury. The transition of phagocytes from suspended cells in the circulation to adherent cells in the periphery is accompanied by dramatic up-regulation of their antimicrobial and cytotoxic capabilities, including generation of ROS and secretion of microbialic compounds and inflammatory mediators, including cytokines (6–11). Despite significant progress in our understanding of the multiple pathways that regulate phagocyte adhesion, characterization of the spatiotemporal determinants of these regulatory components and definition of their integrated functioning with cytoskeletal elements remains an ongoing challenge.

These studies provide evidence for a novel role for PLD in the regulation of phagocyte adhesion.

**FIGURE 5.** Inhibition of PLD results in reduction of adhesion-associated F-actin levels in macrophages. Human PMN were incubated in 1.0% ethanol (■) or buffer control (□) for 5 min, then added to plastic tissue-culture plates. At 5, 15, or 30 min, samples were lysed in buffer containing 0.4 mM TRITC-phalloidin and 0.1% Triton X-100, as detailed in Materials and Methods. Following staining for 1 h, samples were centrifuged and the resultant pellets were extracted with methanol for 20 h, followed by determination of TRITC fluorescence in the extracts. The F-actin content is expressed as the mean ± SEM of the relative fluorescence units (RFU) and represents the data from three identical experiments, each performed in triplicate. The differences between control and ethanol-treated samples at 5, 15, and 30 min are significant at p < 0.01.

**FIGURE 6.** Reduction in PLD1 by RNA interference is associated with inhibition of macrophage adhesion. A. RAW 264.7 macrophages were incubated with buffer (lane 1), FuGENE6 lipofection medium alone (lane 2) scrambled control siRNA (lane 3), or siRNA to murine PLD1 (lane 4). The concentrations of purified siRNA oligonucleotides were 200 nM in FuGENE6, as described in Materials and Methods. After 8 h, cells were washed, replated with fresh medium without siRNAs, and incubated for 5 days, then analyzed by SDS-PAGE and Western blotting with rabbit polyclonal Ab to PLD1, with detection by ECL. B. RAW 264.7 macrophages were incubated in buffer alone (Untreated), scrambled siRNA (SCR-siRNA), or siRNA to PLD1 (PLD1-siRNA). On day 5, macrophages were radiolabeled with [3H]lyso-PAF, and PLD activity during adhesion to plastic was determined at the indicated times. C. Aliquots of the macrophage samples from B were analyzed for adhesion to tissue culture plastic by calcein fluorescence. D. Macrophages were treated as in B and C (Un, untreated; Sc, scrambled siRNA; P1, PLD1-siRNA). On day 5, macrophages were incubated with diC8-PA (100 μM) or buffer control for 15 min, before determination of adhesion to tissue culture plastic for 5 or 15 min. Results in each panel are representative of three experiments. Data in B–D represent the mean ± SEM of triplicate determinations from the three experiments. The differences between samples treated with PLD1-siRNA are significantly different from control at each time point (p < 0.03), denoted by (+), except for those samples in D that were also treated with PA, which did not differ from the controls.

First, adhesion of primary human neutrophils and macrophages, as well as human or murine myelomonocytic cell lines, was associated with rapid and sustained stimulation of PLD activity. Second, this increase in PLD activity occurred in response to adhesion to chemically diverse surfaces, including tissue culture plastic as well as the ECM proteins, FN, collagen, and fibrinogen. Third, PLD1 colocalized with actin filaments at the adhesive interface, in proximity to the focal contact protein,
cells were mechanically detached, followed by rapid readhesion. Detachment stimulated PLD activity, and RNAi-induced suppression of PLD2 reduced readhesion. Our data complement this work by extending it to primary phagocytes (neutrophils and macrophages) under physiologic conditions of adherence to ECM proteins, by demonstrating that adhesion itself was accompanied by stimulation of PLD activity, and that reduction in PLD1 inhibited this process. Our current studies have been restricted to PLD1, due to limitations in specificity of the Ab for human PLD2 and difficulty in suppressing this isoform by RNAi in RAW cells. Taken together, the data of Powner et al. (61) and the current study support a model in which PLD enzymes regulate adhesion in diverse cell types.

Increases in PLD activity may contribute to several biochemical mechanisms that promote adhesion. These include stimulation of Pi(4,5)P_2 synthesis (with the resultant provision of substrate for PI3K and PI-PLC, and activation of actin polymerization), as well as stimulation of multiple isoforms of PKC by both direct and indirect routes (via PA and DAG, respectively) (24–37). Of note, PLD-mediated adhesion of RBL-2H3 cells is dependent on PA-induced stimulation of PI4P5-K, with resultant generation of Pi(4,5)P_2 (61). In Dictyostelium discoideum, inhibition of PLD with 1-butanol markedly reduces actin polymerization and Pi(4,5)P_2 synthesis, both of which are reversed by exogenous PA (89). In addition, PA activates several other signaling pathways, including Ras and PTKs, which may promote adhesion through stimulation of type 1 PI3K (90–94). Finally, of particular relevance to our results, Tool et al. (95) demonstrated that addition of synthetic, cell-permeant diC8-PA promoted CD11b/CD18-dependent adhesion of eosinophils. Further studies will be required to define which of these potential mechanisms contribute to PLD’s enhancement of adhesion in phagocytes. Of note, preliminary studies with human embryonic kidney cells indicate that the activation of PLD during adhesion, and the dependence of early stages of adhesion on PLD activity are not restricted to leukocytes (our unpublished data).

It is important to emphasize that the current study focused on the initial 60 min of adhesion of suspended phagocytes to surfaces coated by ECM proteins or to tissue culture plastic. Additional studies will be required to characterize the role of PLD in later adhesive events and under different experimental systems that mirror the diversity of adhesive phenotypes, including homotypic and heterotypic cell-cell adhesion. In fact, whereas inhibition of PLD1 reduced the level of adhesion within the first hour, preliminary data suggest that over extended intervals of several days, PLD1-deleted cells exhibit increased cell spreading (our unpublished data). Thus, PLD may be intricately involved in the complex relationships between cell adhesion, spreading, and motility, all of which require dynamic rearrangements of the actin cytoskeleton.

We are currently working on improved technical approaches to more clearly define the kinetic relationships between localization and activation of PLD, actin polymerization, and cell adhesion/deadhesion. Improvements in temporal resolution are required due to the rapidity of adhesion-induced PLD activity. Maximal PLD activity is exhibited within 2–5 min of contact with the adhesive surface, and the current endpoint assay for PA and PEt generation is difficult to reproduce in replicate form at shorter intervals.

The prominent effects of adhesion on PLD activity, and conversely, the important role for PLD1 in the initial stages of adhesion, is another example of the mechanistic parallels between phagocytosis and adhesion (1, 3, 79, 80). Phagocytosis is accompanied by activation of both PLD1 and PLD2 (47). Optimal levels

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**FIGURE 7.** PLD1-depleted macrophages exhibit dysregulated adhesion-induced actin polymerization. RAW 264.7 macrophages were incubated with control scrambled siRNA (A and B) or siRNA to PLD1 (C and D). On day 5, cells were adhered to glass chamber slides for 15 min at 37°C, followed by fixation in paraformaldehyde and permeabilization in acetone. Samples were stained with polyclonal Ab to PLD1, with detection via Texas Red-conjugated anti-rabbit IgG secondary Ab. Actin filaments were stained with Alexa 488-labeled phalloidin. Confocal microscopy was used to analyze cells at the adhesion interface (A and B) or 3 μm above the surface (C and D). At least 25 cells were imaged for each condition, and data are representative of four identical experiments. E, RAW macrophages incubated with buffer (untreated), lipofection medium, scrambled control siRNA, or siRNA to PLD1 were adhered to tissue culture plastic for 5 or 15 min. F-actin content was determined via the TRITC-phalloidin assay and data expressed as RFU/10^6 adherent macrophages to correct for differences in adhesion between the samples. Data represent the mean ± SEM from three identical experiments, each performed in triplicate. Differences between PLD1-depleted samples and scrambled control are statistically significant at p < 0.01 (*) or p < 0.05 (+).

paxillin. Fourth, reductions in PLD activity by chemical inhibitors or specific siRNA-induced knockdown of PLD1 resulted in significant inhibition of phagocyte adhesion, and reduced levels of actin filaments. Fifth, addition of cell-permeable PA restored control levels of adhesion to macrophages which had been depleted of endogenous PLD1 by RNAi. Although exogenous administration of PA is not equivalent to its focal generation by PLD activity, the use of cell-permeable lipids has been used previously to provide evidence for the involvement of specific lipid species, including PA, and/or specific lipid-modifying enzymes, including PLD (61, 87, 88).

While this manuscript was in preparation, Powner et al. (61) reported that activation of PLD2 is coupled to adhesion of the RBL-2H3 cell line. Their protocol differed in that the adherent...
of phagocytosis require both PLD isoforms, but only PLD1 localizes to the nascent phagosome in human macrophages (47). Similarly, PLD activity was strongly stimulated during cell adhesion, and inhibition of PLD1 reduced the level of adhesion. Furthermore, PLD1 colocalized with actin filaments at the adhesion interface, both in the cortical ring and at focal contacts. The reductions of adhesion in PLD1-inhibited cells are accompanied by decreased levels of cellular F-actin and dysregulation of actin filaments at the adhesive surface of the cell. We are currently developing methods to characterize these processes in real-time and to comprehensively assess the effects of PLD1 inhibition on the number and strength of focal contacts.

These studies suggest that, in addition to promoting the process of adhesion itself, PLD may contribute to the enhancement of phagocyte responses that occur following the transition from the suspended to the adherent state. For example, in contrast to PMN in suspension, adherent neutrophils exhibit a greatly magnified respiratory burst, that is significantly enhanced in its amplitude and duration (6–11). In this way, adhesion can be considered a “priming” event which converts a cell from a minimally responsive state to one that is biochemically prepared for robust activation by diverse stimuli. This transition from the relatively resting phenotype of suspended phagocytes to the adhesion-induced primed state is likely to be highly relevant to innate immune defense and inflammation. We hypothesize that adhesion-associated activation of PLD and its colocalization with the actin cytoskeleton may contribute to those phagocyte antimicrobial mechanisms which have been demonstrated to require stimulation of PLD and dynamic actin rearrangements, including the respiratory burst and granule secretion. Furthermore, this model suggests that PLD may be involved in other actin-dependent functions that are involved in the transition from a resting phagocyte to an innate immune effector cell, including spreading and motility. The diverse roles for PLD in a wide range of phagocyte functions suggests that it may be an important target for therapeutic modulation to promote antimicrobial activity and limit deleterious inflammatory damage to host tissues.

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