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J Immunol (2004) 173 (4): 2615–2623.

<https://doi.org/10.4049/jimmunol.173.4.2615>

Phospholipases D1 and D2 Coordinately Regulate Macrophage Phagocytosis¹

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Phagocytosis is a fundamental feature of the innate immune system, required for antimicrobial defense, resolution of inflammation, and tissue remodeling. Furthermore, phagocytosis is coupled to a diverse range of cytotoxic effector mechanisms, including the respiratory burst, secretion of inflammatory mediators and Ag presentation. Phospholipase D (PLD) has been linked to the regulation of phagocytosis and subsequent effector responses, but the identity of the PLD isoform(s) involved and the molecular mechanisms of activation are unknown. We used primary human macrophages and human THP-1 promonocytes to characterize the role of PLD in phagocytosis. Macrophages, THP-1 cells, and other human myelomonocytic cells expressed both PLD1 and PLD2 proteins. Phagocytosis of complement-opsonized zymosan was associated with stimulation of the activity of both PLD1 and PLD2, as demonstrated by a novel immunoprecipitation-in vitro PLD assay. Transfection of dominant-negative PLD1 or PLD2 each inhibited the extent of phagocytosis (by 55–65%), and their combined effects were additive (reduction of 91%). PLD1 and PLD2 exhibited distinct localizations in resting macrophages and those undergoing phagocytosis, and only PLD1 localized to the phagosome membrane. The COS-7 monkey fibroblast cell line, which has been used as a heterologous system for the analysis of receptor-mediated phagocytosis, expressed PLD2 but not PLD1. These data support a model in which macrophage phagocytosis is coordinately regulated by both PLD1 and PLD2, with isoform-specific localization. Human myelomonocytic cell lines accurately model PLD-dependent signal transduction events required for phagocytosis, but the heterologous COS cell system does not. *The Journal of Immunology*, 2004, 173: 2615–2623.

Phagocytosis is a central cellular response of innate immunity that is essential to host defense against infection and neoplastic proliferation, wound healing, and tissue remodeling (1–5). Phagocytosis initiates a diverse range of antimicrobial/cytotoxic responses, including generation of the respiratory burst, secretion of inflammatory mediators and Ag presentation. The regulation of phagocytosis has been studied in primary macrophages and neutrophils, myelomonocytic cell lines, and nonphagocytic cells, such as COS monkey fibroblasts, induced to heterologously express phagocytic receptors (1, 2, 6–12). It has proven challenging to decipher the signaling events that regulate phagocytosis and to distinguish the mechanisms required for particle ingestion from those pathways that are critical for subsequent effector responses (2, 5). In particular, our understanding of the spatial and temporal integration of these regulatory signal transduction events has remained limited.

Phagocytosis of both complement- and Ab-opsonized targets, as well as unopsonized particles, is associated with activation of phospholipase D (PLD)³ (6, 13–17). Inhibition of the extent of

phagocytosis by multiple structurally and mechanistically distinct chemical inhibitors of PLD supports an essential role for this lipase in the process of ingestion itself. However, the potential for non-specific effects of these chemical inhibitors cannot be excluded. In addition, the identity of the PLD isoform(s) involved and the spatio-temporal details of activation are unknown. The PLD superfamily is comprised of enzymes of diverse functions that perform essential roles in intracellular signaling and the metabolism of phospholipids and nucleic acids (18–20). This ubiquitous enzyme family is found in viruses, bacteria, fungi, plants, and mammals, and is characterized by a conserved motif, HXXK₁DX₆G(G/S), which encompasses the catalytic HKD triad (21–23). Two mammalian PLDs have been identified and each exhibits complex regulation in vivo and in vitro (18, 19, 24, 25). PLD1 is activated by GTPases of the Rho, Ral, and ADP ribosylation factor families, as well as by protein kinase C (PKC); and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) serves as an essential cofactor (24). PLD2 also requires PI(4,5)P₂, but in contrast to PLD1, exhibits relatively high in vitro activity in the absence of protein cofactors, and appears to be primarily subject to negative regulation (25). However, recent data support a role for both ADP ribosylation factor GTPases and PKC in stimulated activation of PLD2 (26, 27). Due to difficulties in purification and their complex regulation, the isoform-specific functions of mammalian PLDs have been difficult to establish. Furthermore, information on the expression and subcellular localization of PLD1 and PLD2 in primary cells and tissues has remained limited (28–30).

The major questions addressed in this study were: 1) Which isoforms of PLD regulate macrophage phagocytosis?; 2) What are the kinetic and spatial determinants of this regulation?; and 3) Do heterologous model systems accurately reproduce the key features of PLD activation during phagocytosis?

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Received for publication February 13, 2004. Accepted for publication June 1, 2004.

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¹ These studies were supported by National Institutes of Health GM62302 and AI055916, and Veterans Affairs Merit Review grants to D.J.K.

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³ Abbreviations used in this paper: PLD, phospholipase D; COZ, complement-opsonized zymosan; CR3, complement receptor 3; DN, dominant negative; dTHP-1, differentiated THP-1; IP, immunoprecipitate; LAMP-1, lysosome-associated membrane protein-1; MDM, monocyte-derived macrophages; PA, phosphatidic acid; PET, phos-

phatidylethanol; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; TR, Texas Red.

Materials and Methods

Materials

Unless otherwise stated, materials were from previously published sources (14, 16, 31–34). Polyclonal Abs to PLD1 or PLD2 were raised by immunizing rabbits with specific peptides for each protein (four for PLD1, two for PLD2), as previously described (28). mAb to lysosome-associated membrane protein-1 (LAMP-1) was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Oregon Green- and Texas Red (TR)-conjugated secondary Abs, Texas-Red-conjugated zymosan, and latex beads (3- μ m diameter) were from Molecular Probes (Eugene, OR). Glass chamber slides were from Fisher Scientific (Hampton, NH).

Preparation of macrophages

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. PBMC were isolated and cultured in Teflon wells for 5 days in RPMI 1640 (pH 7.4) with 20% fresh autologous serum as previously described (14, 31). Macrophages 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 experimental manipulations on macrophage viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with naphthol blue-black stain (14, 31). Purity and viability of macrophage were >95% by Wright staining and trypan blue exclusion, respectively.

Cell lines and transfection

THP-1 and U937 human promonocytic leukemia cell lines and the COS-7 monkey fibroblast cell line were from American Type Culture Collection (Manassas, VA). The K562 human erythroleukemia cell line expressing functional complement receptor 3 (K562-CR3) was generously provided by E. J. Brown (University of California, San Francisco, CA). Plasmids encoding GFP fusion proteins of the catalytically inactive PLD mutants (PLD1^{K398R} and PLD2^{K758R}) (28–30) and wild-type PLD1 and PLD2 were kindly provided by M. A. Frohman (State University of New York, Stony Brook, NY). Plasmids were transfected into THP-1 or K562-CR3 via electroporation. A total of 10 μ g of plasmid DNA was added to cells (5 \times 10⁶ cells/ml) in RPMI 1640 in a final volume of 0.4 ml. The cuvettes were placed on ice for 5 min, then samples were electroporated at 200 V, 960 μ F in a Bio-Rad Gene Pulser (Hercules, CA). Samples were immediately diluted 5-fold in RPMI 1640, 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, and incubated at 37°C for 24 h. Transfected THP-1 cells were differentiated to a macrophage-like phenotype (differentiated THP (dTHP)-1 cells) by incubation in 1,25 dihydroxyvitamin D₃ (100 nM), IFN- γ (1000 U/ml), and retinoic acid (1 μ M) for 48 h before analysis (16). Transfection efficiencies were ~10% for THP-1 cells and 45% for K562-CR3 cells.

PLD activity in intact cells

THP-1 cells were radiolabeled with [³H]oleate (5 μ Ci/ml) for 18 h in Iscove's medium, 10% FBS, 1% penicillin/streptomycin at 37°C, 7.5% CO₂. Cells were washed 3 \times in H/S buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 0.7 mM MgCl₂, 0.5 mM EGTA, 10 mM glucose, 1 mg/ml BSA), and resuspended in the same. A total of 10⁶ cells/sample were incubated with 1.0% ethanol for 2 min to permit detection of the PLD-specific transphosphatidylated product, phosphatidylethanol (PEt), followed by complement-opsonized zymosan (COZ, particle:cell ratio of 10:1), PMA (100 nM), or buffer control, in a total volume of 500 μ l. Reactions were conducted for 30 min at 37°C, and terminated by addition of 5 vol of chloroform:methanol (2:1, v/v). Lipids were extracted, dried under N₂, and analyzed by TLC in an ethylacetate:isooctane:acetic acid (9:5:2) solvent system, and PEt was identified by comigration with pure standard (16, 33, 35). [³H]PEt cpm were quantitated by liquid scintillation spectrometry, and counts normalized for the total amount of ³H-labeled phospholipid in each experiment. [³H]cpm comigrating with PEt were determined for each set of samples in the absence of ethanol, and these background counts were subtracted from each data point.

Determination of isoform-specific PLD activity

Monocyte-derived macrophages (MDMs) or dTHP-1 cells in H/S buffer were incubated with COZ, 100 nM PMA, or buffer control for 15 min at 37°C. Reactions were terminated by washing in ice-cold PBS. Cells were solubilized in lysis buffer, containing 0.3% Triton X-100 and 0.5% octyl glucoside in H/K buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 2 μ M leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT) (16, 32), by incubation for 1 h on ice. Following

centrifugation at 14,000 \times g for 15 min at 4°C, to pellet the insoluble fraction, supernatants were precleared by incubation with protein A-Sepharose for 120 min at 4°C. Lysates were centrifuged at 1,000 \times g for 5 min at 4°C, and supernatants were incubated with rabbit polyclonal Abs to PLD1, PLD2, or control preimmune serum for 5 h at 4°C, followed by an additional 1-h incubation with 50 μ l of 10% protein A-Sepharose. Immunoprecipitates (IPs) were washed in complete lysis buffer, followed by 0.5% octyl glucoside in H/K buffer, then three times with H/K buffer without detergents. PLD activity of the IPs was assayed by addition of mixed lipid substrate vesicles containing phosphatidylethanolamine:PI(4,5)P₂:phosphatidylcholine (PC) in a molar ratio of 16:1.4:1, with 10 uCi/sample of [³H]dipalmitoylphosphatidylcholine and 1.5% ethanol (16, 36). To confirm the efficacy and specificity of immunoprecipitation, samples were also subjected to SDS-PAGE on 8% gels. Following transfer to polyvinylidene difluoride membrane, Western blotting was performed with anti-PLD 1/2 Abs, with detection by ECL.

Phagocytosis assay

Phagocytosis of COZ was determined by modification of a previously described flow cytometry assay (37), in which the target particles are labeled with TR (E_x 595 nm, E_m 615 nm). Phagocytosis was synchronized by centrifuging the COZ onto the THP-1 cells at 200 \times g for 2 min at 15°C, which permits adherence, but not phagocytosis. Phagocytosis was initiated by placing the samples in a 37°C incubator for designated intervals. Ingestion was terminated by treatment with lyticase (300 U/ml) that digests extracellular zymosan (37), with confirmation by confocal microscopy. Expression of the PLD mutants as enhanced GFP (E_x 488 nm, E_m 530 nm) fusion proteins (38) enabled gating on the transfected dTHP-1 cells. Dead cells were excluded via uptake of propidium iodide (PI). Phagocytosis was quantitated as the mean fluorescence intensity at 615 nm (emission maximum for TR) in the GFP-transfected cells. There was no difference in the level of phagocytosis of dTHP-1 cells transfected with GFP control vector compared with untransfected cells, indicating that electroporation and plasmid transfection did not affect phagocytosis.

Confocal microscopy

Primary human MDMs were adhered to glass chamber slides and incubated with buffer or latex beads (particle:cell ratio of 3:1), followed by fixation in 3.75% paraformaldehyde for 15 min and permeabilization in ice-cold methanol:acetone (1:1) (31, 39). Following incubation with blocking buffer (PBS, 5% BSA, 10% horse serum) for 1 h, polyclonal Abs to PLD1 (1/30 dilution) or PLD2 (1/300 dilution) were added for 1 h, samples were washed repeatedly and incubated with TR-conjugated secondary anti-IgG Ab for 1 h, all at 25°C. In select experiments, mAbs to vesicle-specific protein markers (LAMP-1, lysosomes; early endosomal Ag 1, early endosomes; β coatamer protein, Golgi) were coincubated with the anti-PLD Abs, with subsequent detection with goat anti-murine IgG-Oregon Green 2° Ab. 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 (Oberkochen, Germany).

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 Student's *t* test. Nonparametric evaluation of other data sets was performed with the Mann-Whitney Rank Sum test (40).

Results

Primary human macrophages and myelomonocytic cell lines express both PLD1 and PLD2

The cell and tissue distributions of PLD enzymes are not widely known, and limitations in sensitivity and specificity of the available Abs have yielded conflicting results (16, 41–44). We have used improved anti-peptide Abs to PLD1 and PLD2 (28) to determine their distribution in primary human macrophage and several representative human myelomonocytic cell lines. Human macrophage and the human promonocytic cell lines, THP-1 and U937, express both PLD1 and PLD2 proteins with low levels of PLD2 detected in U937 cells (Fig. 1A). Differentiation of THP-1 and U937 cells to a macrophage-like phenotype by incubation with IFN- γ , 1,25-dihydroxyvitamin D₃, and retinoic acid (16, 45, 46), resulted in increased levels of both PLD1 and PLD2 in U937 cells, but no significant change was noted in THP-1 cells (Fig. 1A). The

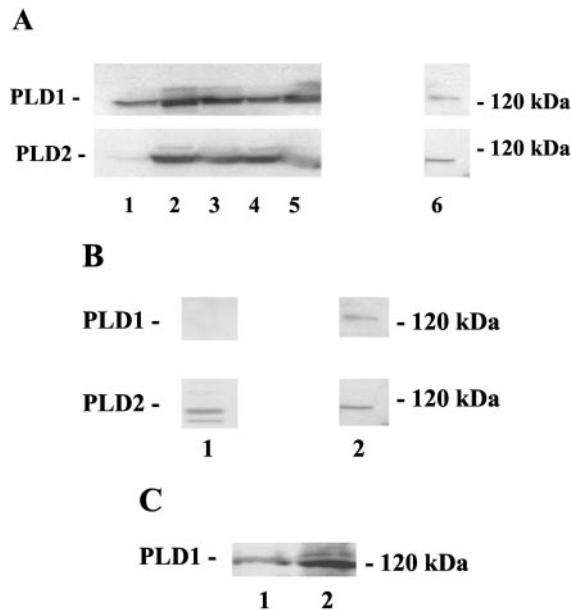


FIGURE 1. Primary human macrophages and myelomonocytic cell lines express both PLD1 and PLD2. *A*, Macrophages (*lane 6*) were derived from blood monocytes by *in vitro* culture for 5 days. U937 (*lanes 1 and 2*), THP-1 (*lanes 3 and 4*), and K562 (*lane 5*) cell lines were incubated in the absence (*lanes 1, 3, and 5*) or presence (*lanes 2 and 4*) of IFN- γ (1000 U/ml), 1,25-dihydroxyvitamin D₃ (100 nM), and retinoic acid (1 μ M) for 72 h. A total of 10⁶ cells were subjected to SDS-PAGE on 8% gels, followed by Western blotting with polyclonal Abs to PLD1 or PLD2 with detection by ECL. *B*, COS (*lane 1*) and primary human macrophages (*lane 2*) were analyzed for PLD1 and PLD2. *C*, Anti-PLD1 Western blot of primary monkey brain (*lane 1*) and baculovirus-expressed recombinant human PLD1 (*lane 2*). Data are representative of three identical experiments.

human K562 erythroleukemia cell line, derived from a patient with chronic myelogenous leukemia, and often used as a cell line model for neutrophils (47), also expressed both PLD isoforms.

In contrast to phagocytes, COS cells express detectable PLD2, but not PLD1 protein

COS are a nonmyeloid (monkey fibroblast) cell line that has frequently been used to study phagocytosis, via transfection of phagocytic receptors, including complement and Fc γ Rs (9–12). Western blot analysis demonstrated that COS cells express abundant PLD2 protein, but no detectable PLD1 protein (Fig. 1*B*). The lack of detectable PLD1 in COS cells was not due to a failure of the Ab to detect monkey PLD1 protein, because endogenous PLD1 was readily detected in primary monkey brain tissue and comigrated with baculovirus-expressed recombinant human PLD1. The lack of PLD1 in COS cells has been previously reported with a different anti-PLD1 Ab (48).

Phagocytic particles stimulated increased levels of PLD1 and PLD2 activity

A diverse array of phagocytic particles, including those opsonized with complement or Ab, as well as unopsonized particles, result in stimulation of PLD activity, as determined in intact macrophage, neutrophils, and myelomonocytic cell lines (6, 13–17). To specifically determine which isoforms of PLD are activated during phagocytosis, we developed an immunoprecipitation *in vitro* PLD assay. THP-1 cells were differentiated to a macrophage-like phenotype by incubation with IFN- γ , 1,25-dihydroxyvitamin D₃, and retinoic acid for 72 h (45, 46). These dTHP-1 cells adhered to tissue culture-treated plastic, expressed numerous pseudopods, and

exhibited increased phagocytic activity, compared with the undifferentiated state. Resting dTHP-1 cells were incubated with buffer control, COZ (particle:cell ratio of 10:1), or 100 nM PMA for 30 min. Cells were solubilized in lysis buffer (0.3% Triton X-100, 0.5% octyl glucoside in H/K buffer) and immunoprecipitated with polyclonal Abs to PLD1, PLD2, or control preimmune serum. To minimize detergent-induced interference with the assay of PLD activity, IPs were washed sequentially in lysis buffer, 0.5% octyl glucoside in H/K, and then detergent-free H/K buffer, before addition of [³H]dipalmitoylphosphatidylcholine vesicles.

Resting cells exhibited low levels of PLD activity in the control and PLD2 IPs, whereas a small amount of PLD1-specific activity was detected (Fig. 2*A*). Addition of COZ resulted in significant stimulation of PLD1 and PLD2 activity compared with the control IP from the same cells, and to the anti-PLD1 and anti-PLD2 IPs from buffer-treated cells. The level of PLD1 activity in COZ-treated samples was 210 \pm 8% of the control value in buffer-treated cells, whereas PLD2 activity was 203 \pm 13% of control (mean \pm range, *n* = 4). These data support the hypothesis that phagocytosis is associated with stimulation of both PLD1 and PLD2. Stimulation of cells with PMA, which is known to activate both PLD1 and PLD2 (18, 27), resulted in increased levels of activity in IPs containing PLD1 (170 \pm 14% of control), as well as PLD2 (163 \pm 7%), supporting the accuracy of this assay (Fig. 2*A*).

Parallel experiments in primary macrophages yielded similar results, with both COZ and PMA resulting in the activation of PLD1 and PLD2. The level of PLD1 activity in COZ-treated primary macrophages was 165 \pm 4% of the control value in buffer-treated cells, whereas PLD2 activity was 206 \pm 5% of control (mean \pm range, *n* = 2). PMA treatment resulted in increased levels of activity in IPs containing PLD1 (223 \pm 24% of control) as well

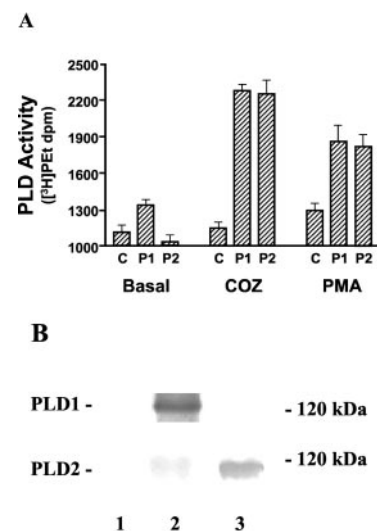


FIGURE 2. Phagocytosis is associated with stimulation of both PLD1 and PLD2. *A*, dTHP-1 macrophages were incubated with buffer (Basal), COZ (particle:cell, 10:1), or PMA (100 nM) for 30 min. Cells were disrupted in lysis buffer, precleared, and then immunoprecipitated with control, preimmune serum (C), or specific rabbit polyclonal Abs to PLD1 (P1) or PLD2 (P2). Following extensive washing of the IPs, PLD activity of each was determined by addition of mixed lipid vesicles containing [³H]PC substrate and 1.0% ethanol. The PLD-specific product, PEt, was isolated by TLC and quantitated by liquid scintillation spectrometry. Data are mean \pm SD from one of four identical experiments, each performed in duplicate. *B*, IPs from control (*lane 1*), PLD1 (*lane 2*), or PLD2 (*lane 3*) samples were subjected to Western blotting with Abs to PLD1 or PLD2, as indicated. Data are representative of results from three identical experiments.

as PLD2 ($196 \pm 8\%$). Thus, stimulation of both dTHP-1 macrophages and primary macrophages with the phagocytic particle, COZ, or the soluble agonist, PMA, resulted in activation of PLD1 and PLD2. Western blotting of the IPs confirmed the specificity of the Abs, (Fig. 2B), as previously reported (28, 49, 50). No PLD1 protein was detected in the anti-PLD2 IPs, and only a minor amount of PLD2 was detected in the anti-PLD1 IPs.

Dominant-negative (DN) PLD1 and PLD2 inhibit phagocytosis

Several studies have demonstrated that structurally distinct chemical inhibitors of PLD-dependent signaling (ethanol, butanol, and 2,3-diphosphoglycerate) reduce the extent of phagocytosis. However, these inhibitory approaches are limited by the potential for nonspecific effects. Furthermore, the chemical inhibitors do not distinguish between PLD1 and PLD2, and thus cannot be used to determine isoform-specific functions of PLD enzymes. To directly evaluate the potential roles of PLD1 and PLD2 in the regulation of phagocytosis, we used catalytically inactive mutants in which lysine of one of the HKD triads (K898 in PLD1 and K758 in PLD2) are replaced by arginine (51). These catalytically inactive PLD mutants have been demonstrated to confer isoform-specific DN phenotypes in several cell types (28–30). However, to date, there is no information on the specific roles of PLD1 or PLD2 in any macrophage function, nor in phagocytosis by any cell type.

Phagocytosis of COZ, a well-established model target particle for phagocytic CRs (2, 14, 16, 52), was determined by modification of a previously described flow cytometry assay in which the target particles are labeled with the fluorophore, TR (37). Extracellular adherent zymosan was removed by digestion with lyticase (37), with efficacy confirmed by confocal microscopy. The DN-PLD mutants (PLD1^{K898R} and PLD2^{K758R}) were expressed as fusion proteins with enhanced GFP to enable gating on transfected macrophage. THP-1 cells transfected with control GFP vector demonstrated no change in phagocytosis compared with untransfected cells, indicating that electroporation and expression of GFP did not affect phagocytosis (Fig. 3A). In contrast, transfection of PLD1^{K898R} resulted in a reduction of phagocytosis of 56% (range 52–60%, $p < 0.01$, $n = 4$) (Fig. 3A). The catalytically inactive PLD2 mutant (PLD2^{K758R}) conferred a similar level of inhibition, with reduction in phagocytosis of 63% (range 59–67%, $p < 0.01$, $n = 4$). Transfection of both PLD1^{K898R} and PLD2^{K758R} resulted in almost complete suppression of phagocytosis, with a reduction of 94% (range 91–98%, $p < 0.001$, $n = 4$). Similar reductions in the level of phagocytosis by DN-PLD1, DN-PLD2, and the combination of both were noted at 5, 15, 30, and 60 min periods of particle ingestion (data not shown). These data are consistent with the hypothesis that both PLD1 and PLD2 contribute nonredundant roles that are essential to optimal phagocytosis. Furthermore, the individual and additive effects of the DN-PLD mutants are consistent with the results of the IP *in vitro* PLD assay that indicated that both PLD1 and PLD2 are stimulated by phagocytic particles.

To further evaluate the hypothesis that both PLD1 and PLD2 participate in the regulation of phagocytosis, we used the same experimental approach in an alternative human myeloid cell line, K562. K562 cells have previously been used to study numerous phagocyte functions, including adhesion, phagocytosis, production of reactive oxidant species, and antimicrobial activity (53–55). We used a variant of K562 cells that stably expresses functional CR3 (Mac-1, CD11b/CD18) and exhibits enhanced phagocytic capacity (47), hereafter termed K562-CR3 cells. K562-CR3 cells were transfected by electroporation with plasmids encoding GFP control protein or GFP fusion proteins of DN-PLD1, DN-PLD2, or both DN mutants. Of note, the transfection efficiency of K562-CR3 cells (45%) was much greater than that of THP-1 (10%) cells.

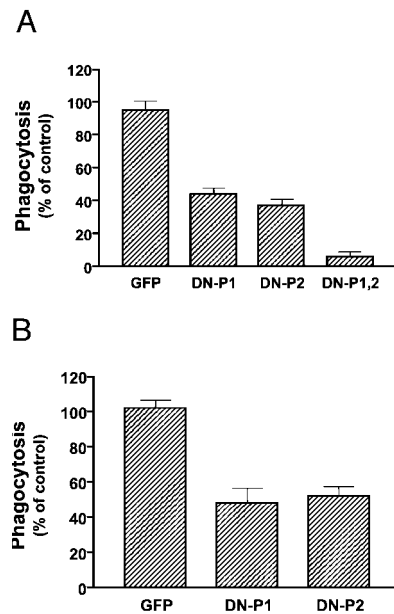


FIGURE 3. DN-PLD1 and PLD2 inhibit phagocytosis. *A*, THP-1 cells were transfected with GFP vector (GFP), or DN mutants of PLD1 (DN-P1), PLD2 (DN-P2), or both (DN-P1,2) via electroporation. Following differentiation, cells were incubated with TR-labeled COZ (at a particle:cell ratio of 4:1) for 30 min at 37°C. Extracellular COZ was removed with lyticase, and phagocytosis by transfected cells was quantitated by flow cytometry. *B*, The effects of DN-PLDs on phagocytosis of K562-CR3 cells was determined as in *A*. Toxicity of the dual transfection for K562-CR3 cells precluded its analysis. Data for each transfected cell population are expressed as the percent phagocytosis of control untransfected cells, and are the mean \pm range from four identical experiments. The phagocytosis index of control cells was 212 ± 16 COZ ingested per 100 THP-1 cells and 188 ± 14 for K562-CR3.

Similar to the results with THP-1 cells, both DN-PLD1 and DN-PLD2 significantly inhibited the extent of phagocytosis (Fig. 3B), confirming the hypothesis that both PLD isoforms function in the regulation of phagocytosis in myelomonocytic cells. Unlike THP-1 cells, the effect of transfection of both DN-PLDs could not be evaluated in K562-CR3 cells, due to a high level of toxicity in cotransfected cells. This difference may be due to the much greater efficiency of transfection in K562-CR3 cells.

PLD1 and PLD2 exhibit distinct differences in subcellular localization in primary human macrophages, in both the resting and activated states

The spatial and temporal determinants of signal integration are crucial parameters of regulatory networks. Phagocytosis provides a unique opportunity to characterize these spatio-temporal aspects of coordinate regulation, because it provides a defined locus, the phagosome, upon which multiple biochemical networks converge. Thus, macromolecular signaling complexes are assembled, and then modified throughout the maturational process that transforms the nascent phagosome to a microbicidal phagolysosome. Studied at a population level, the data from the DN-PLD mutants are consistent with a crucial role for both PLD1 and PLD2 in the regulation of phagocytosis. Our hypothesis is that PLD1 and PLD2 subserve unique nonredundant roles in phagocytosis. To test this hypothesis, we used laser scanning confocal microscopy to determine the subcellular localization of endogenous PLD1 and PLD2 in primary human macrophages, in both the resting state, as well as during phagocytosis. Our primary goal was to determine whether spatial and temporal distinctions could be made between the PLD isoforms in resting and activated phagocytes.

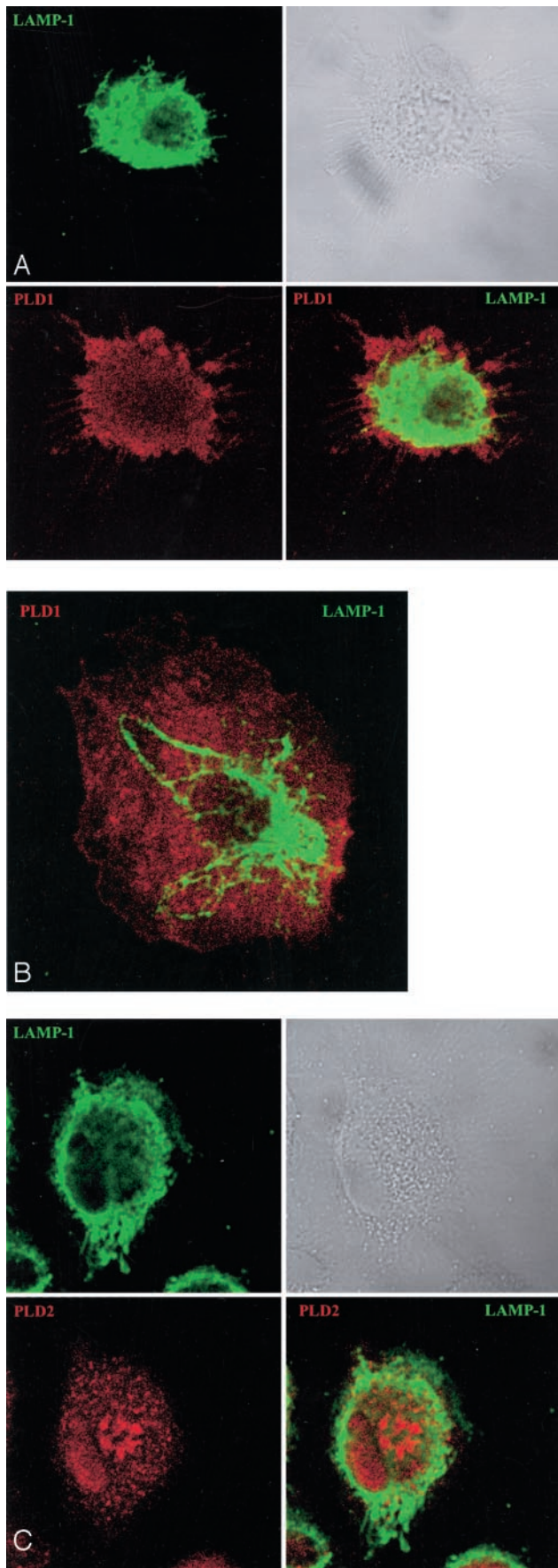


FIGURE 4. PLD1 and PLD2 exhibit distinct subcellular localizations in resting human macrophages. Primary MDMs were adhered to glass chamber slides for 3 h, washed, fixed in paraformaldehyde, and permeabilized in

In resting macrophages, PLD1 localized to both the cell cortex as well as punctate intracellular structures (Fig. 4, *A* and *B*). The cortical distribution is consistent with the plasma membrane localization proposed from biochemical studies of phagocytes (16, 32, 48, 56–58). The intracellular PLD1 is likely vesicular in origin, in agreement with data from several cell types (28, 49, 58). PLD2 localized to intracellular vesicles (especially in the perinuclear region) and the nucleus in resting macrophages (Fig. 4*C*). The late endosomal/lysosomal marker protein, LAMP-1, showed a minor degree of colocalization with PLD1 and PLD2 in resting macrophages.

Macrophages underwent synchronized phagocytosis of 3- μ m latex beads and confocal microscopy was performed at serial intervals following particle addition. Latex beads were used, rather than COZ, because the latter exhibited strong nonspecific staining. PLD1 was significantly enriched on nascent phagosomes, including the forming phagocytic cup and newly fused phagosomes (Fig. 5*A*). The presence of high levels of PLD1 on the phagosome persisted at 15 min (Fig. 5*B*), at which time a significant percentage of total cellular PLD1 staining was located on the phagosomal membrane. The accumulation of LAMP-1 at 15 min is consistent with the maturation of the phagosome to a phagolysosome (59, 60). At 30 min following ingestion, the level of phagosomal PLD1 was decreased (Fig. 5*C*), whereas at 60 min, no phagosomal PLD1 was detectable (Fig. 5*D*). In summary, PLD1 strongly localized to nascent phagosomes and early phagolysosomes for the first 30 min following ingestion.

In marked contrast to PLD1, the subcellular distribution of PLD2 remained essentially unchanged during phagocytosis (Fig. 6). The localization of PLD2 in phagocytosing macrophages was indistinguishable from that of resting cells, namely, intracellular vesicles and the nucleus. The most prominent vesicular staining for PLD2 was again noted in the perinuclear region. No PLD2 was detected at the phagosomal membrane within 5–60 min of particle addition. Thus, despite a similar requirement for PLD1 and PLD2 for optimal phagocytosis, these PLD isoforms differed markedly in subcellular localization in resting macrophages, and only PLD1 became physically associated with the phagosomal membrane from its inception at the cell surface to its maturation to a phagolysosome.

Of note, in macrophages undergoing phagocytosis, there was increased colocalization of PLD1 and PLD2 with LAMP-1 in perinuclear vesicles that were distinct from phagosomes (Figs. 5*D* and 6, *C* and *D*). The presence of LAMP-1 suggests that these vesicles are lysosomes or late endosomes, but further characterization will be required for identification. PLD activity has previously been detected in lysosomes (61), but, to our knowledge, the isoform of PLD responsible for this activity has not been defined.

Discussion

Characterization of the molecular mechanisms that regulate phagocytosis is fundamental to our understanding of vital physiologic and pathologic processes, including host immunity, inflammation, wound healing, and tissue remodeling. Within the immune system, phagocytosis is critically important to both innate responses and their linkage to adaptive immunity. Furthermore, many of the biochemical pathways that regulate phagocytosis, including PLD, also function in the antimicrobial/cytotoxic responses of macrophages,

methanol:acetone, as detailed in *Materials and Methods*. Samples were blocked and incubated with primary Abs; rabbit polyclonal Abs to PLD1 (*A* and *B*) or PLD2 (*C*), and mAb to LAMP-1. Anti-PLD Abs were detected with TR-conjugated anti-rabbit IgG 2^o Ab, and LAMP-1 was detected with anti-murine IgG-Oregon Green 2^o Ab. The phase-contrast and merged images are shown in *A* and *C*. Data are representative of 25 cells examined from each of six identical experiments.

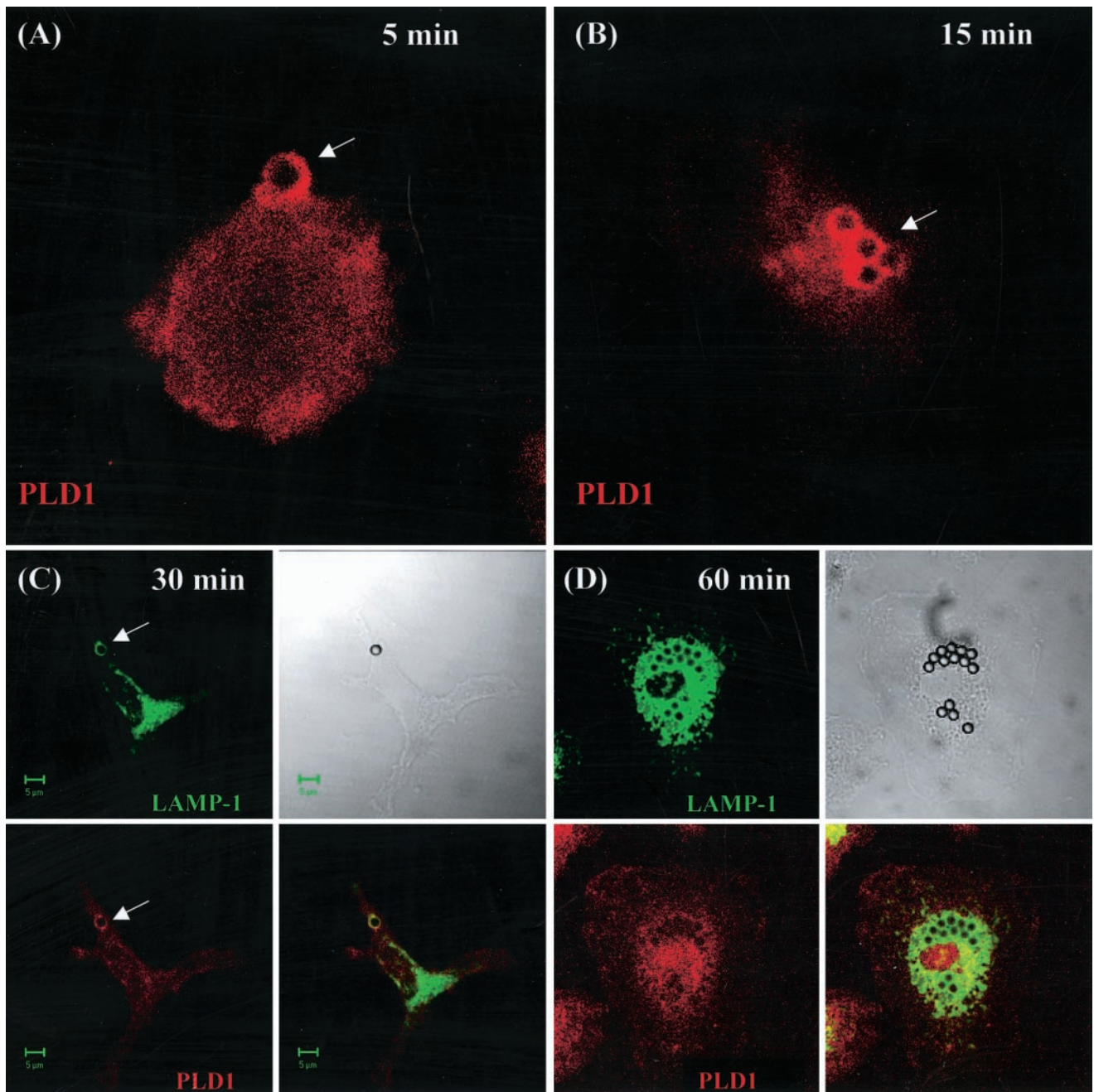


FIGURE 5. PLD1 localizes to early phagosomes. MDMs were adhered to glass chamber slides and incubated with buffer or latex beads (particle:MDM ratio of 3:1). Synchronized phagocytosis was accomplished by centrifugation at $200 \times g$ for 2 min at 15°C , followed by warming in a 37°C incubator for the indicated times. Samples were fixed and processed for confocal microscopy as described in *Materials and Methods*. PLD1 strongly localized to phagosomes (arrows) at 5 (A), and 15 (B), and 30 min (C). At 60 min, PLD1 was not detected on the phagosome surface (D). Data are representative of 25 cells examined from each of six identical experiments.

monocytes, neutrophils, and dendritic cells. In this study, we present several novel aspects of the regulation of phagocytosis by PLD enzymes. First, primary human macrophages and myelomonocytic cell lines express both PLD1 and PLD2 proteins. Second, phagocytosis is associated with stimulation of the enzymatic activities of both PLD1 and PLD2. Third, DN mutants of PLD1 or PLD2 inhibit phagocytosis, and their combined effects are additive. Of note, transfection of PLD1^{K898R} and PLD2^{K758R} essentially eliminated the phagocytic capacity of dTHP-1 macrophages. Fourth, PLD1 and PLD2 exhibit distinct subcellular localizations in both resting and phagocytosing primary human macrophages. Fifth, COS cells, which have been used as a heterologous system for

the analysis of receptor-mediated phagocytosis, do not accurately model the coordinate regulation of phagocytosis by PLD1 and PLD2 that is exhibited by primary phagocytes and myelomonocytic cell lines, because they express only PLD2 protein.

There is limited data on the distribution and subcellular localization of mammalian PLD enzymes in primary cells. In bovine adrenal chromaffin cells and rat cerebellar granule cells, PLD1 is localized predominantly to the plasma membrane (28, 62), whereas in rat liver homogenates PLD1 is detected in the both the Golgi apparatus and plasma membrane upon subcellular fractionation (49). In cell lines, PLD1 has been localized to intracellular membranes (Golgi, endosomes, lysosomes, and nucleus) as well as

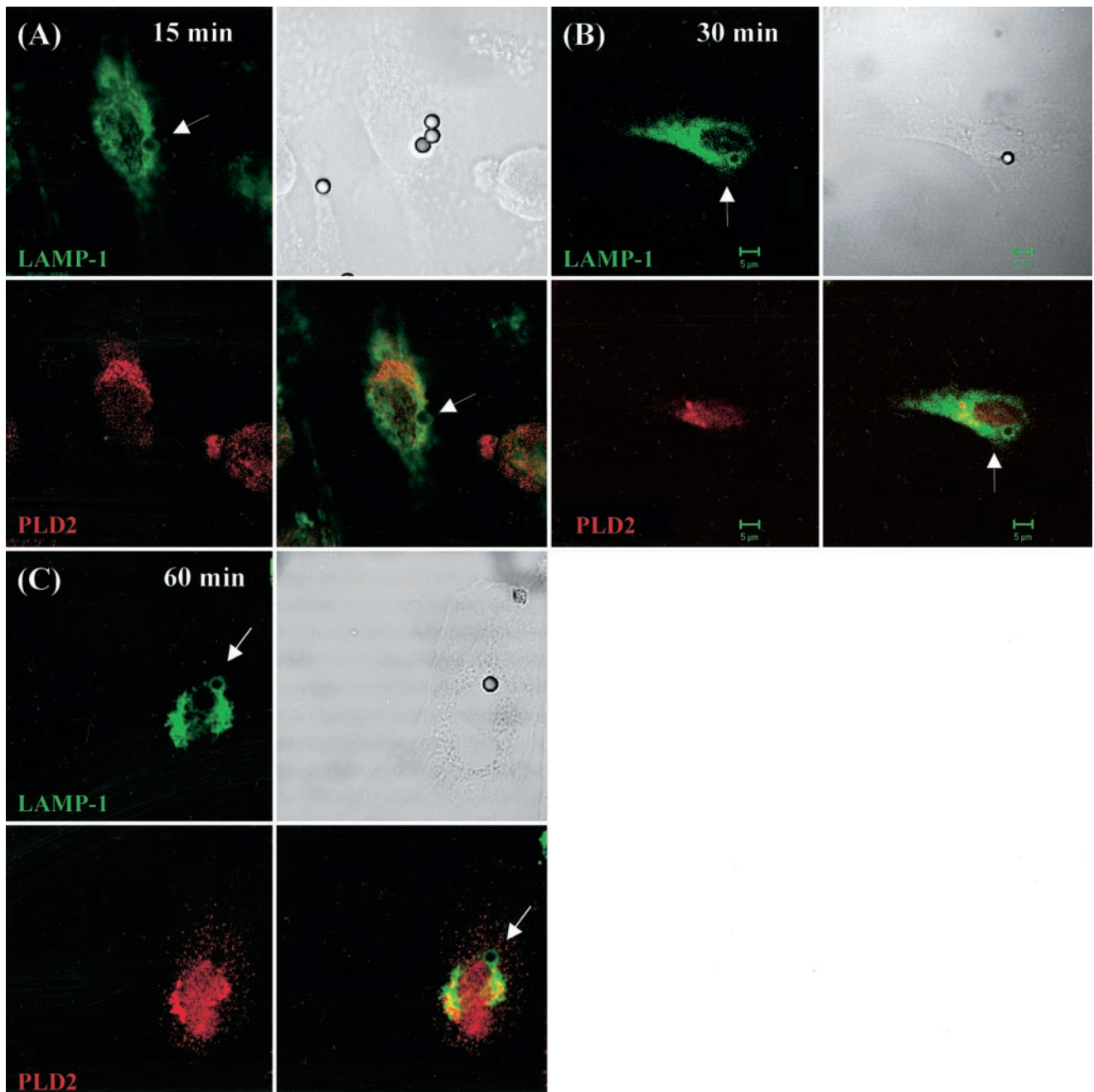


FIGURE 6. PLD2 does not localize to macrophage phagosomes. MDMs underwent synchronized phagocytosis of latex beads, for the indicated times. Samples were processed for confocal microscopy and stained with polyclonal anti-PLD2, detected by TR-conjugated 2°Ab, and mAb to LAMP-1, detected by Oregon Green-2°Ab. The distribution of PLD2 closely resembled that in resting cells and no localization of PLD2 to the phagosome (arrows) was detected at any time between 5 and 60 min. Data are representative of 25 cells examined from each of five identical experiments.

the plasma membrane (28, 49, 57, 63). Transfection of chromophore- or epitope-tagged PLD1 has demonstrated a similar range of subcellular distributions, along with evidence of activation-associated translocation of PLD1 from secretory vesicles to the plasma membrane (58, 64–66). Conflicting evidence exists concerning whether overexpression of PLD proteins may (49, 50, 66) be accompanied by aberrant subcellular localization.

PLD2 has been localized to the plasma membrane of primary mouse cardiomyocytes (67) and to the sarcoplasmic reticulum membrane of rat cardiomyocytes (68). However, in primary mouse cerebellar granule neurons, PLD2 exhibited a primarily intracellular punctate (vesicular) distribution (69). Subcellular fractionation of rat liver demonstrated predominant localization of endogenous PLD2 in

the Golgi apparatus and light membranes (70). In cell lines, endogenous PLD2 has been demonstrated in the plasma membrane, Golgi apparatus, and nucleus (50, 63, 67). Overexpressed PLD2 has been demonstrated in the plasma membrane as well as intracellular vesicles and the nucleus (18, 64, 67). Like PLD1, PLD2 has been demonstrated to undergo agonist-induced translocation between membrane compartments (25, 67, 71, 72).

To our knowledge, this report represents the first characterization of the identity and subcellular localization of PLD enzymes in primary human phagocytic leukocytes and myelomonocytic cell lines. The presence of both PLD1 and PLD2 in these cells raises the question of their stimulation under physiologic conditions. Using a novel immunoprecipitation-in vitro PLD assay, we demonstrated that

phagocytosis is coupled to the stimulation of both PLD1 and PLD2. This activation is likely causally linked to the ingestion process itself, because DN mutants of either PLD1 or PLD2 significantly inhibited the level of phagocytosis. In previous studies, these DN mutants have exhibited isoform-specific suppression of PLD1 and PLD2 (28–30). In the current report, the additive inhibition of phagocytosis by combined transfection of PLD1^{K898R} and PLD2^{K758R} supports the hypothesis that the catalytically inactive PLD mutants confer isoform-specific suppression.

The suppression of macrophage phagocytosis by PLD1^{K898R} and PLD2^{K758R} is consistent with a model in which PLD1 and PLD2 exhibit unique nonredundant roles in innate immune cells. Further support for this hypothesis is provided by the differences in spatial distribution of the PLD isoforms, particularly in activated macrophages. In resting macrophages, PLD1 is localized to the plasma membrane and intracellular vesicles, whereas PLD2 is found on intracellular vesicles and in the nucleus.

Because PLD1 and PLD2 cycle between membrane compartments in stimulated cells (25, 58, 64–67, 71, 72), it is possible that their distribution in resting human macrophages could be influenced by cellular activation events associated with adherence itself. Upon binding of a particle, PLD1 is rapidly recruited to the nascent phagosome and remains strongly enriched on its surface throughout the maturation to a phagolysosome. The localization of PLD1 to the phagosome persists for 30 min following particle addition, but is no longer detectable at 60 min. Although it is required for optimal phagocytosis, PLD2 was not detectable on the phagosomal membrane at any time within the first 60 min of particle addition. In fact, its cellular distribution in phagocytosing macrophages remained indistinguishable from resting cells at all time points analyzed in this study.

Although further studies will be required to define the unique functions of PLD1 and PLD2 in macrophages and other phagocytic leukocytes, the striking differences in their subcellular localizations suggest distinct possibilities during phagocytosis. Griffiths and colleagues (73) have recently detected phosphatidic acid (PA), the product of PLD-mediated catalysis and a documented membrane fusogen, on the phagosomal membrane. Our data support the hypothesis that PLD1 is responsible for the generation of phagosomal PA and may directly contribute to the maturation of phagosomes to phagolysosomes via sequential fission and fusion events with vesicles of the endosomal-lysosomal system (59, 60). Conversely, PLD2 may function in cellular responses that contribute to phagocytosis from more distant sites, such as stimulation of PKC, which itself then translocates to the phagosome (74). Both PLD1 and PLD2 have been linked physically and functionally to the actin cytoskeleton (16, 75–80), and each may contribute to actin's role in phagocytosis, but at different cellular sites. Such complementary, but distinctive, functions for PLD1 and PLD2 have recently been characterized in colonic epithelia (28) and mast cells (64).

Nonphagocytic cells, such as COS monkey fibroblasts, have served as important model systems in the characterization of several fundamental features of phagocytosis (9–12). However, the present data indicate that they do not accurately model the coordinate regulation of macrophage phagocytosis by PLD1 and PLD2, and suggest that caution is appropriate in considering their use for the study of biochemical pathways and physiologic functions that are closely coupled to PLD-dependent generation of PA.

Acknowledgments

We thank our colleagues in the Inflammation Program at the University of Iowa for valuable advice and critique, and Michael A. Frohman and Eric J. Brown for their generous provision of reagents. We are very grateful for the valuable assistance of Justin Fishbaugh and Gene Hess with the

FACS analysis, and Randy Nessler and Christopher Thompson with the confocal microscopy.

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