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*J Immunol* 2008; 180:6027-6034; doi: 10.4049/jimmunol.180.9.6027

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Phospholipase D1 Plays a Key Role in TNF-α Signaling

Swaminathan Sethu, Grecia Mendez-Corao, and Alirio J. Melendez

The primary characteristic features of any inflammatory or infectious lesions are immune cell infiltration, cellular proliferation, and the generation of proinflammatory mediators. TNF-α is a potent proinflammatory and immuno-regulatory cytokine. Decades of research have been focused on the physiological/pathophysiological events triggered by TNF-α. However, the signaling network initiated by TNF-α in human leukocytes is still poorly understood. In this study, we report that TNF-α activates phospholipase D1 (PLD1), in a dose-dependent manner, and PLD1 is required for the activation of sphingosine kinase and cytosolic calcium signals. PLD1 is also required for NFκB and ERK1/2 activation in human monocytes. Using antisense oligonucleotides to reduce specifically the expression of PLD isozymes showed PLD1, but not PLD2, to be coupled to TNF-α signaling and that PLD1 is required to mediate receptor activation of sphingosine kinase and calcium transients. In addition, the coupling of TNF-α to activation of the phosphorylation of ERK1/2 and the activation of NFκB were inhibited by pretreating cells with antisense to PLD1, but not to PLD2, thus; demonstrating a specific requirement for PLD1. Furthermore, use of antisense oligonucleotides to reduce expression of PLD1 or PLD2 demonstrated that PLD1 is required for TNF-α-induced production of several important cytokines, such as IL-1β, IL-5, IL-6, and IL-13, in human monocytes. These studies demonstrate the critical role of PLD1 in the intracellular signaling cascades initiated by TNF-α and its functional role for coordinating the signals to inflammatory responses. The Journal of Immunology, 2008, 180: 6027–6034.

The primary characteristic features of any inflammatory or infectious lesions are immune cell infiltration, cellular proliferation, and the generation of proinflammatory mediators. TNF-α is a potent proinflammatory and immuno-regulatory cytokine. Decades of research have been focused on the physiological/pathophysiological events triggered by TNF-α. However, the signaling network initiated by TNF-α in human leukocytes is still poorly understood. In this study, we report that TNF-α activates phospholipase D1 (PLD1), in a dose-dependent manner, and PLD1 is required for the activation of sphingosine kinase and cytosolic calcium signals. PLD1 is also required for NFκB and ERK1/2 activation in human monocytes. Using antisense oligonucleotides to reduce specifically the expression of PLD isozymes showed PLD1, but not PLD2, to be coupled to TNF-α signaling and that PLD1 is required to mediate receptor activation of sphingosine kinase and calcium transients. In addition, the coupling of TNF-α to activation of the phosphorylation of ERK1/2 and the activation of NFκB were inhibited by pretreating cells with antisense to PLD1, but not to PLD2, thus; demonstrating a specific requirement for PLD1. Furthermore, use of antisense oligonucleotides to reduce expression of PLD1 or PLD2 demonstrated that PLD1 is required for TNF-α-induced production of several important cytokines, such as IL-1β, IL-5, IL-6, and IL-13, in human monocytes. These studies demonstrate the critical role of PLD1 in the intracellular signaling cascades initiated by TNF-α and its functional role for coordinating the signals to inflammatory responses. The Journal of Immunology, 2008, 180: 6027–6034.
Materials and Methods

Cell line

U937 cells were cultured in RPMI 1640 (Life Technologies), supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, and 10 mg/ml streptomycin at 37°C, 6.8% CO₂ in a water-saturated atmosphere. The cells were treated with IFN-γ (Bender Wien Ltd; 200 ng/ml) for 16 h for them to be differentiated into a monocytic phenotype.

Primary monocytes

Mononuclear cells were isolated from heparinized fasting venous blood by Ficoll-Hypaque centrifugation as previously described (27). The 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered carefully on 15 ml of Ficoll-Hypaque gradient and centrifuged at 500g, without brakes, at room temperature for 30 min. The mixed mononuclear band was aspirated and the cells were washed three times in phenol red RPMI 1640 medium containing 100 U/ml penicillin, 100 × g/ml streptomycin, and 2 mM glutamine and suspended in a known volume. Leukocyte count was performed on a Coulter counter and then cells were plated (5–7 × 10⁵ cells) in 6-well culture plates in RPMI 1640 medium. Incubation was conducted at 37°C for 2 h in 5% CO₂/95% air, after which, nonadherent cells were removed by washing the wells twice with RPMI 1640, and the remaining adherent cells were grown in the culture medium supplemented with 10% FCS and 2 mM glutamine for 2 days. The cells were used after 2 days of culture.

Cell viability, determined by trypan blue exclusion, was ~94% in all experiments.

Antisense oligonucleotides

Antisense oligonucleotides were purchased from 1st Base (Singapore); 24mers were synthesized, capped at either end by the phosphorothioate linkages (first two and last two linkages), and corresponded to the reverse complement of the first 8 amino acids for either PLD1 or PLD2. The sequences of the oligonucleotides were:

5’ CCGTGGCCTCGTTTTCCGTACAT 3’ for PLD1 and 5’ GAG GCCTTCAGGGGGTGCCGAT 3’ for PLD2.

U937 cells were incubated in 10 μM oligonucleotide for a total of 36 h (20 h before and then for the duration of culture with IFN-γ). Primary monocytes, 12 h in culture, were incubated in 10 μM oligonucleotide for a total of 36 h.

Measurement of PLD activity

PLD activity was measured as previously described (21, 26), using the transphosphatidylation assay. Briefly, U937 cells were labeled (10⁶ cells/ml) with ³²P-halopatetic acid (5 μCi/ml; Amersham Biosciences) in the cell culture medium for 16 h. Following washing, the cells were incubated at 37°C for 15 min in RPMI 1640 medium containing ethanol (0.3% final). Following TNF-α (PeproTech) 10 ng/ml stimulation for 0, 2, 5, 10, 20, 30, and 60 min at 37°C, cells were then lysed and lipids extracted by Bligh-Dyer phase separation. The accumulated phosphatidylethanol was assayed as described previously (21).

Fluorescent microscopy

To study re-localization patterns of PLD isoforms, normal resting cells were stimulated by TNF-α over a time scale (0, 2, 5, and 10 min). After TNF-α stimulation, the suspended cells were fixed in 4% paraformaldehyde and deposited on microscope slides using a cytospin centrifuge; they were then permeabilized for 5 min in 0.1% Triton X-100 in PBS. The permeabilized cells were blocked for nonspecific binding with 5% FCS for 10 min at room temperature. Fluorescent labeling was done by incubating the cells with goat-polyclonal PLD1 (Santa Cruz Biotechnology), goat-polyclonal PLD2 (Santa Cruz Biotechnology), and an irrelevant goat-polyclonal (Santa Cruz Biotechnology), primary Abs for 1 h at room temperature. The cells were washed with PBS and then incubated with appropriate secondary Abs (anti-goat IgG-FITC conjugate) for an hour. The cell-laden slides were then washed, cover slips were mounted, and the cells preserved using Fluorsave reagent (Calbiochem). To a set of cells, irrelevant control Abs plus the secondary Abs were added as control. Staining was visualized with an inverted fluorescence Leica DM IRB microscope and recorded by a Leica DC 300F digital camera; pictures were analyzed with the Leica IMS500 Image Manager software.

Immunoprecipitation of PLD

PLD1 and PLD2 were immunoprecipitated from cell lysates before Western blot analysis of the desired proteins. Goat polyclonal Ab (2 μg), either anti-PLD1 or anti-PLD2 (Santa Cruz Biotechnology), were incubated with 50 μl of 50% Protein A-agarose and 450 μl of PBS, for 2 h on a rocking platform at 4°C to form precipitating complexes, a goat-polyclonal against ARF-1 was used as an irrelevant control for immunoprecipitation. Then, the Ab and Protein A-agarose mix was washed to remove unbound Ab. Following this, 500 μl of cell lysate containing 200 μg protein was mixed with the precipitating (Ab:protein A-agarose) complex and placed in a tumbler at 4°C for 4 h. Following incubation, the precipitating complex was centrifuged and washed, before addition of Lamelli buffer for loading on to 8% polyacrylamide gels (SDS-PAGE).

Western blot

Following TNF-α stimulation at various time points (0, 5, 10, and 30 min), the cell lysates were prepared using RIPA lysis buffer and then protein concentrations were estimated. Proteins were then resolved on 12% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to polyvinylidene difluoride membranes (Millipore). The polyvinylidene difluoride membranes were handled as per the manufacturer’s instructions. The membranes were incubated with the relevant primary Abs (p-p44/42, p-p38 – Cell Signalling, GAPDH – Santa Cruz Biotechnologies) and appropriate HRP-conjugated secondary Abs (Sigma-Aldrich) at room temperature. The membranes were washed with washing buffer (1% Tween 1% PBS) and bands were visualized by autoradiography using ECL Western blotting detection system (Amersham Biosciences).

Cytosolic calcium measurement

Cytosolic calcium was measured as described previously (27). Briefly, cells were loaded with 1 μg/ml Fura-2 AM in PBS, 1.5 mM Ca²⁺, and 1% BSA. After a 30 min recovery of excess sequestration by dilution and centrifugation, the cells were resuspended in 1.5 mM Ca²⁺-supplemented PBS and warmed to 37°C in the cuvette. After the basal line was obtained, the cells were stimulated by the addition of TNF-α. Fluorescence was measured at 340 and 380 nm and the background-corrected 340:380 ratio was calibrated as previously described (27).

Sphinogosine kinase (SphK) activity in cell extracts

Cells (2 × 10⁵) per sample were stimulated with TNF-α, as above. Following TNF-α stimulation the cells were lysed and the cell extracts were assayed for SphK activity. SphK activity was measured as described previously (27). Briefly, the system is based upon the SphK-catalyzed transfer of the γ-phosphosphate group of ATP (using a mixture of cold ATP and [γ³²P]ATP, 1 μCi/sample; Amersham Biosciences) to a specific substrate, then the products were separated and analyzed as previously described (27).

NFkB activity assay

NFkB activity was assayed from cells, which were either pretreated or not with the antisense against PLD1 or PLD2 and stimulated with TNF-α (10 ng/ml). NFkB activity was analyzed using the EZ-Detect transcription factor kit (Pierce) following the manufacturer’s instructions. Briefly, this kit is based on an ELISA format, provided in a 96-well format with oligonucleotides containing the consensus binding sequences for the transcription factor coated on the wells. Cell extracts are incubated in the wells, and bound transcription factors are then detected by a specific primary Ab; a HRP-conjugated secondary Ab is then used to detect the bound primary Ab. The enzymatic product can be measured with any standard plate reader.

Cytokine detection

Cells (2 × 10⁵) pretreated or not with antisense oligos were stimulated by the addition of TNF-α (10 ng/ml) for 24 h. Following stimulation, the supernatants were collected at the indicated time points and stored at −20°C until used. IL-1β, IL-6, IL-5, and IL-13 levels in the supernatants were evaluated using ELISA (BD Pharmingen), following the manufacturer’s instructions with lower detection limits of ≤10 pg/ml.

Statistical analysis

Statistical differences between control and treated cells were calculated using Student’s t-test. A statistical difference of at least 95% (p < 0.05) was considered significant.

Results

TNF-α induces PLD activity in human monocytic cells and subcellular re-localization of PLD1

We first investigated whether TNF-α would induce the activity of PLD in the human monocytic cell line used. TNF-α stimulated a
FIGURE 1. TNF-α triggers PLD activity and PLD1 translocation. A, PLD activity was measured from unstimulated cells (Basal) and cells stimulated with TNF-α 10 ng/ml (TNF-α) for 0, 2, 5, 10, 20, 30, and 60 min. Results are the mean ± SD of triplicate measurements from three separate experiments. B, PLD activity was measured following stimulation with different concentrations of TNF-α for 30 min. Results are the mean ± SD of triplicate measurements from three separate experiments. C, Fluorescent microscopy of cells stimulated with TNF-α 10 ng/ml for the times indicated in the figure, immune-stained for PLD1; an irrelevant goat-polyclonal primary Ab was used for staining controls (Ab control). The results shown are typical from three separate experiments. D, Fluorescent microscopy of cells stimulated with TNF-α 10 ng/ml for the times indicated in the figure, immune-stained for PLD2; an irrelevant goat-polyclonal primary Ab was used for staining controls (Ab control). The results shown are typical from three separate experiments.

rapid increase in PLD activity, which was detectable even after 2 min of TNF-α stimulation (Fig. 1A), and this was a dose-dependent response with maximal activity at 10 ng of TNF-α; however, activity at 2 and 5 ng of TNF-α was quite robust (Fig. 1B). Since both PLD1 and PLD2 (PLD isoforms) have been found to be expressed in the human monocytic cells used (21), we decided to look at the subcellular localization of each of the two isoforms and whether TNF-α stimulated their relocalization, as a means to start dissecting the specific isoform(s) activated by this cytokine. Fluorescent microscopy-derived results, in our study, reveal that in resting cells, both PLD isoforms have a general cytosolic localization and that TNF-α induces the re-localization of PLD1, but not PLD2, to the cells’ periphery (Fig. 1, B and C). This would suggest that the PLD isoform activated by TNF-α is, potentially, PLD1.

TNF-α stimulates PLD1

As both isoforms for PLD are expressed in U937 cells, experiments were performed to examine their respective roles; in particular, their activities following TNF-α stimulation. To do this, specific antisense oligonucleotides were designed against each of the PLD isoforms to specifically knock-down the expression of each enzyme (i.e., antisense to PLD1 and antisense to PLD2). We have previously shown that U937 cells are sensitive to antisense manipulation (21). IFN-γ primed cells were treated with antisense oligonucleotides, and PLD activity was assayed in unstimulated cells to measure basal levels of activity, or after stimulation with TNF-α. The specificity of the antisense oligonucleotides on relative PLD enzyme expression was checked by Western blot analysis (Fig. 2A). In this study, it was found that, in cells treated with antisense to PLD1, there was a reduction in PLD1 immunoreactivity (a reduction of 81 ± 5% was quantified by densitometry, from three separate experiments), whereas PLD2 immunoreactivity was unaffected. Conversely, in cells treated with antisense to PLD2, there was a reduction in PLD2 immunoreactivity (a reduction of 85 ± 10% was quantified by densitometry, from three separate experiments), whereas PLD1 immunoreactivity remained unchanged. Each antisense oligonucleotide, therefore, acted as an internal control for the other. Further controls were performed by the immunoprecipitation of ARF1, with a goat primary Ab, as control of immunoprecipitation experiments, as well as of antisense specificity.

Treatment of cells with the antisense oligonucleotide to PLD1 resulted in no change in basal PLD activity. However, following TNF-α stimulation, the increase in PLD activity was significantly reduced, compared with the control cells (p < 0.01) (Fig. 2B). The reduction in the increase after TNF-α activation was 80 ± 5% in cells treated with antisense PLD1, compared with control cells, and was proportional to the observed reduction in protein expression by Western blot analysis. In contrast, treatment of cells with the antisense oligonucleotide to PLD2 significantly reduced basal PLD activity (p < 0.01). TNF-α-mediated activation of PLD was marginally reduced in cells treated with the antisense to PLD2, but this reduction was entirely accounted for by the reduction in basal levels; the increment over basal was identical in control (untreated) cells and those pretreated with PLD2 antisense oligonucleotide (Fig. 2B), demonstrating that, at least in this system, TNF-α stimulation specifically activates PLD1.

PLD1, and not PLD2, couples TNF-α to the activation of SphK, cytosolic calcium transients, and NF-κB activation

Previously, we have shown that TNF-α, in human monocytes, results in a SphK-dependent release of calcium from intracellular stores (27). In this study, we show that SphK activity and cytosolic calcium responses triggered by TNF-α depend on PLD1.

Pretreating cells with the antisense oligonucleotide to PLD1 to knock-down isozyme expression significantly reduced the activation of sphingosine kinase, following TNF-α stimulation, by 78 ±
NF-κB and its subsequent coupling to cytosolic calcium signals and NF-κB controls.

PLD2 had no effect on the calcium transients, compared with through PLD1 activation, but TNF-α-induced p38 phosphorylation was not inhibited when the cells were pre-treated with antisense PLD2 (TNF-α a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).

FIGURE 2. Use of antisense oligonucleotides to specifically knock-down the expression of either PLD1 or PLD2 demonstrates that only PLD1 is coupled to TNF-α signaling. A. Western blot analysis of immunoprecipitates of either PLD1, PLD2, and of ARF1 (as irrelevant control), to assess expression of either isozyme in cells, following treatment for 36 h with antisense oligonucleotides (10 μM) specific for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD 2), and control cells (Control); blots also show immunoprecipitates. The results shown are typical from three separate experiments. B. PLD activity following TNF-α (10 ng/ml) stimulation, for 30 min, in cells pretreated with 10 μM antisense oligonucleotides for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2). 1-Basal (Basal control); 2-TNF-α stimulation (TNF-α control); 3-Basal in cells pretreated with a PLD1 (Basal a.s.PLD1); 4-TNF-α stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).

One of the key transcription factors that trigger the generation of many proinflammatory molecules is NFκB. In this study, we show that the TNF-α-triggered activation of p50 and p65-NFκB in human monocytes is substantially reduced in cells pretreated with the antisense oligonucleotide against PLD1, whereas pretreatment with a PLD2 antisense oligonucleotide had no observed effect on NFκB activity (Fig. 3C).

These results suggest that PLD1 is upstream of SphK activity and its subsequent coupling to cytosolic calcium signals and NFκB activation.

FIGURE 3. Coupling of TNF-α to downstream intracellular signaling pathways requires PLD1 and not PLD2. A. TNF-α-induced sphingosine kinase activity is dependent on PLD1. Following TNF-α (10 ng/ml) stimulation, cells were harvested at given time points indicated in the figure to measure sphingosine kinase activity. Sphingosine kinase activity was assayed from basal control cells (Basal control), following TNF-α stimulation in control cells (TNF-α) and in cells pretreated with antisense oligonucleotides (10 μM) for either PLD1 (TNF-α a.s.PLD1) or PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements and from three separate experiments and Student’s t test p values (*, p < 0.01). B. Intracellular cytosolic calcium changes following TNF-α stimulation: responses were compared in control cells and cells pretreated with antisense oligonucleotides (10 μM) to either PLD1 or PLD2. Traces shown are: blue, control cells treated with TNF-α 10 ng/ml (TNF-α Control); black, cells pretreated with antisense to PLD1 (TNF-α a.s.PLD1); and red, cells pretreated with antisense to PLD2 (TNF-α a.s.PLD2). The arrow marks the addition of TNF-α. Traces are typical from three separate experiments. C. TNF-α triggered p50 and p65 NFκB activity; p50 and p65-NFκB activity was measured in resting cells (Basal) or following TNF-α 10 ng/ml stimulation for 30 min in cells pretreated or not with antisense oligos: 1-Basal (Basal control); 2-TNF-α stimulation (TNF-α control); 3-Basal in cells pretreated with a PLD1 (Basal a.s.PLD1); 4-TNF-α stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).

TNF-α is functionally coupled to ERK1/2 phosphorylation through PLD1 activation, but TNF-α-induced p38 phosphorylation is independent of PLD activity

TNF-α-triggered activation of MAPKs has important functional consequences for several cellular responses, including for the generation of cytokines. Our study demonstrates a critical role for PLD1 in mediating the TNF-α-induced phosphorylation of ERK1/2. Treatment of cells with the antisense oligonucleotide to PLD1 resulted in the inhibition of the phosphorylation levels of ERK1/2 triggered by TNF-α (Fig. 4A). In contrast, treatment of cells with the antisense oligonucleotide to PLD2 did not alter TNF-α-induced ERK1/2 phosphorylation, compared with control cells (Fig. 4A). To evaluate the relevance of PLD1 in p38 kinase activity in the TNF-α-triggered signaling pathway, we looked at the phosphorylation of p38 induced by TNF-α. We found that TNF-α did indeed trigger p38 phosphorylation in the human monocyte cells; however, in contrast to ERK1/2 phosphorylation, the phosphorylation of p38 was not inhibited when the cells were pretreated with antisense oligonucleotide to PLD1 (Fig. 4B). Similarly, pretreatment of cells with the antisense oligonucleotide to
PLD2 did not alter the TNF-α-induced p38 phosphorylation (Fig. 4B).

Taken together these results suggest that PLD1 is upstream of ERK1/2, but TNF-α-mediated p38 phosphorylation is independent of PLD activity. It has been suggested that PLD activity may be downstream of ERK1/2 activation (28–31). To investigate this, experiments were conducted in cells pretreated with MEK and p38 inhibitors, and the TNF-α-triggered PLD activity was measured. The results shown in Fig. 4C demonstrate that, at least in this system, the TNF-α-induced PLD activation is independent of MAPK activity, as both inhibitors failed to moderate its activity. The inhibitors were shown to be working properly as both of them inhibited the TNF-α-induced phosphorylation of their target proteins (Fig. 4, A and B).

PLD1 is required for TNF-α-triggered cytokine generation

TNF-α is capable of amplifying the inflammatory response by promoting the generation and release of several proinflammatory cytokines and chemokines. In this study, we show that TNF-α stimulates IL-1β, IL-5, IL-6, and IL-13 in unstimulated (Basal) and after stimulation with TNF-α 10 ng/ml (TNF-α), pretreated or not with the antisense oligos: 1-Basal (Basal control); 2-TNF-α stimulation (TNF-α control); 3-Basal in cells pretreated with a.s.PLD1 (Basal a.s.PLD1); 4-TNF-α stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).

FIGURE 4. TNF-α-triggered ERK1/2 phosphorylation is PLD1 dependent, whereas p38 phosphorylation is PLD independent. A, TNF-α triggered ERK1/2 phosphorylation; Western blot analysis of phosphorylated ERK1/2 following TNF-α (10 ng/ml) stimulation for the time course indicated in the figure in untreated control cells (TNF-α Control); in cells pretreated with the PLD1 antisense (TNF-α a.s.PLD1); in cells pretreated with the PLD2 antisense (TNF-α a.s.PLD2); and in cells pretreated with the ERK inhibitor, PD98059 at 50 μM (TNF-α PD98059). Results shown are typical of three separate experiments. GAPDH was probed for loading control. B, TNF-α triggered p38 phosphorylation; Western blot analysis of phosphorylated p38 following TNF-α (10 ng/ml) stimulation for the time course indicated in the figure in untreated control cells (TNF-α Control); in cells pretreated with the PLD1 antisense (TNF-α a.s.PLD1); in cells pretreated with the PLD2 antisense (TNF-α a.s.PLD2); and in cells pretreated with a p38 kinase inhibitor, SB203580 at 10 μM (TNF-α SB203580). Results shown are typical of three separate experiments. GAPDH was probed for loading control. C, TNF-α-triggered PLD activity is independent of ERK or p38 activation. PLD activity in unstimulated cells (Basal); in cells stimulated with TNF-α 10 ng/ml (TNF-α); in cells pretreated with ERK inhibitor, PD98059 at 50 μM before stimulation with TNF-α 10 ng/ml (PD98059 + TNF-α); and in cells pretreated with p38 kinase inhibitor, SB203580 (10 μM), before stimulation with TNF-α 10 ng/ml (SB203580 + TNF-α). Results are the mean ± SD of triplicate measurements and from three separate experiments.

FIGURE 5. TNF-α-triggered cytokine release is inhibited in cells pretreated with the PLD1 antisense. Measurements of IL-1β, IL-5, IL-6, and IL-13 in unstimulated (Basal) and after stimulation with TNF-α 10 ng/ml (TNF-α), pretreated or not with the antisense oligos: 1-Basal (Basal control); 2-TNF-α stimulation (TNF-α control); 3-Basal in cells pretreated with a.s.PLD1 (Basal a.s.PLD1); 4-TNF-α stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).
FIGURE 6. TNF-α activates the PLD1 pathway in primary human monocytes. A, PLD activity was measured following stimulation with different concentrations of TNF-α for 30 min. Results are the mean ± SD of triplicate measurements from three separate experiments. B, Fluorescent microscopy of cells stimulated with TNF-α 5 ng/ml for the times indicated in the figure, immune-stained for PLD1; an irrelevant goat-polyclonal primary Ab was used for staining controls (Ab control). The results shown are typical from three separate experiments. C, Fluorescent microscopy of cells stimulated with TNF-α 5 ng/ml for the times indicated in the figure, immune-stained for PLD2; an irrelevant goat-polyclonal primary Ab was used for staining controls (Ab control). The results shown are typical from three separate experiments. D, Western blot analysis of immunoprecipitates of PLD1, PLD2, and ARF1, following treatment for 36 h with antisense oligonucleotides (10 μM), specific for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2), and control cells (Control). The results shown are typical from three separate experiments. E, PLD activity following TNF-α (5 ng/ml) stimulation in cells pretreated with 10 μM antisense oligonucleotides for either PLD1 (a.s.PLD1) or PLD2 (a.s.PLD2). 1-Basal (Basal control); 2-TNF-α (5 ng/ml) stimulation (TNF-α control); 3-Basal in cells pretreated with a.s.PLD1 (Basal a.s.PLD1); 4-TNF-α (5 ng/ml) stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α (5 ng/ml) stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01). F, Following TNF-α (5 ng/ml) stimulation, cells were harvested at given time points indicated in the figure to measure sphingosine kinase activity. Sphingosine kinase activity was assayed from basal control cells (Basal control), following TNF-α stimulation in control cells (TNF-α control) and in cells pretreated with antisense oligonucleotides (10 μM) for either PLD1 (TNF-α a.s.PLD1) or PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements and from three separate experiments and Student’s t test p values (*, p < 0.01). G, Intracellular cytosolic calcium changes following TNF-α (5 ng/ml) stimulation; responses were compared in control cells and cells pretreated with antisense oligonucleotides (10 μM) to either PLD1 or PLD2. Traces shown are: solid line, control cells treated with TNF-α 5 ng/ml (TNF-α Control); dotted line, cells pretreated with antisense to PLD1 (TNF-α a.s.PLD1); and broken line, cells pretreated with antisense to PLD2 (TNF-α a.s.PLD2). The arrow marks the addition of TNF-α. Traces are typical from three separate experiments. H, Measurements of IL-1β, IL-5, IL-6, and IL-13 in unstimulated (Basal) and after stimulation with TNF-α 5 ng/ml (TNF-α), pretreated or not with the antisense oligos: 1-Basal (Basal control); 2-TNF-α stimulation (TNF-α control); 3-Basal in cells pretreated with a.s.PLD1 (Basal a.s.PLD1); 4-TNF-α stimulation in cells pretreated with a.s.PLD1 (TNF-α a.s.PLD1); 5-Basal in cells pretreated with a.s.PLD2 (Basal a.s.PLD2); and 6-TNF-α stimulation in cells pretreated with a.s.PLD2 (TNF-α a.s.PLD2). Results are the mean ± SD of triplicate measurements from three separate experiments and Student’s t test p values (*, p < 0.01).
SphK and to cytosolic calcium responses (Fig. 6, F and G). Moreover, in the primary human monocytes PLD1 was also essential for the TNF-α-mediated release of cytokines (Fig. 6H).

Taken together, the data presented in this report strongly suggest that PLD1 is a key signaling molecule that mediates TNF-α-triggered proinflammatory responses in human monocytes.

Discussion
In this study, we have shown that TNF-α is functionally coupled to PLD1, but not PLD2, in IFN-γ primed U937 cells, as well as in human primary monocytes, even though both enzymes are expressed in these cells. We further show that PLD1, and not PLD2, is required for TNF-α-mediated activation of the sphingosine kinase-mediated intracellular calcium responses, the activation of key transcription factors and cytokine generation.

Understanding the intracellular signal transduction mechanisms that regulate TNF-α-mediated responses has profound implications, not the least of which is to identify novel molecules as potential therapeutic targets. There is overwhelming evidence for believing that TNF-α is associated with a variety of inflammatory conditions in several diseases (32–47). Efforts are being made to find novel ways to balance TNF-α levels or TNF-α-induced responses in several diseases, including rheumatoid arthritis and systemic lupus erythematosus. Regulation of molecules involved in the TNF-α-triggered signaling pathways has gained attention in this regard, as a variety of signaling molecules such as PDE4, p38 MAP-kinase, and NfκB inhibitors are being studied in clinical trials (4), but have met with undesired side effects so far. More efficient therapies may become available by elucidation of the molecular mechanisms, and the role of key molecules, involved in the TNF-α-triggered signaling events. Thus, we investigated the role of PLD isoforms in the TNF-α-mediated intracellular and effector responses in human monocytes.

Our results show that TNF-α triggers PLD activity and that PLD1 (but not PLD2) is rapidly translocated from an acytosolic distribution to the plasma-membrane periphery. We and others have previously shown that PLD plays a role in inflammatory signals (30) and may stimulate MAPK phosphorylation events (48–51), or prevent protein de-phosphorylation (51).

The cells used in our study expressed both PLD isoforms (PLD1 and PLD2) (21); however, this study shows a selective re-localization of PLD1 to the cell membrane after TNF-α stimulation. This would suggest that TNF-α induces the selective activation of PLD1. However, as there are many reports indicating the stimulation of isoform-specific PLDs in different cells, responding to distinct stimuli (21, 28–31), we decided to further investigate the specific isoform activated by TNF-α. We used antisense oligonucleotides to specifically knockdown the expression of either PLD1 or PLD2. Our results using the antisense demonstrate that TNF-α specifically activates PLD1 but not PLD2.

To further our studies of the role of PLD1 in TNF-α-mediated intracellular signaling events, we looked at the role of PLD in signals triggered by TNF-α. We have previously shown that, in these cells, TNF-α activates and uses sphingosine kinase to mediate calcium release from internal stores (27). In this study, we show that in cells pretreated with the antisense against PLD1, the TNF-α-mediated SphK activity and cytosolic calcium responses are substantially inhibited, whereas in the cells pretreated with the antisense against PLD2, these TNF-α-mediated responses remain intact. This is further proof for the specificity of PLD1 in the TNF-α-mediated responses.

MAPKs are vital regulators and/or amplifiers of extracellular stimuli leading to cellular functions; they are regulated by sequential phosphorylation of their preceding kinase-family members (8). Our study shows that the TNF-α-stimulated ERK1/2 phosphorylation is dependent on PLD1; however, the phosphorylation of p38 is not. Interestingly, several studies have reported receptor-coupled activation of ERK1/2 to be not only independent of PLD activity, but that PLD activity was actually dependent on ERK1/2 activity (29). We show here that, at least in human monocytes, TNF-α signals in a very different way, i.e., TNF-α-stimulated PLD activity is upstream of ERK1/2 phosphorylation, whereas p38 phosphorylation is independent of PLD activity. This contrasts with an elegant report by Bechoua and Daniel (28), that showed that fMLP-triggered p38 activation in HL-60 cells was dependent on PLD activity, whereas ERK1/2 phosphorylation was not. To clarify these potential contradictions, and to establish the molecular specificity in our system, we used MEK and p38 inhibitors and looked at PLD activity triggered by TNF-α. Our results showed that neither inhibition of MEK nor p38 had any effect on TNF-α-mediated PLD activity; the inhibitors were shown to be working correctly as they inhibited their respective targets.

As the NfκB and ERK1/2 pathways appear to be dependent on PLD activity, we went on to measure the TNF-α-triggered proinflammatory cytokine production, and showed that the antisense to PLD1 substantially blocks the various cytokines measured.

Taken together, the data presented here suggest that, in human monocytes, TNF-α mobilizes intracellular calcium through the coupling of PLD1 to SphK1, so far this is the first example of this pathway to be shown in cytokine signaling. The fact that TNF-α needs to couple to PLD1 to trigger calcium release from internal stores may, at least in part, explain the role of PLD1 in TNF-α-mediated NfκB activation, as it has been shown that calcium amplitude and/or modulation is required for NfκB activation (52, 53, 54), moreover, it has also been shown that the product of SphK-activity (S1P) can activate NfκB in U937 cells (55). In this study, we also demonstrate that PLD1 is required for the phosphorylation/activation of ERK1/2 MAPKs. The immediate product of PLD1 is phosphatidic acid. Previous studies have shown that Raf-1 can be activated by phosphatidic acid (48, 49). Our finding that ERK1/2 is downstream of PLD1 is, therefore, consistent with this in vitro work (48, 49). Interestingly, it has long been established that members of the MAPK family can phosphorylate the IκB α kinase complex, which in turn leads to the activation of NfκB (56, reviewed in Ref. 54).

TNF-α stimulation in myeloid cells triggers a number of effector functions, including the generation of several cytokines. The novel intracellular signaling pathway demonstrated here appears to be functionally interactive/associated with these. In the study reported here, silencing PLD1 reduced or abolished the ability of TNF-α to mobilize calcium from intracellular stores. In addition, the knockdown of PLD1 significantly reduced the activation of key transcription factors, such as NfκB and ERK1/2 MAPKs. It is of interest that these transcription factors play major roles in the inflammatory responses, by triggering cytokine and chemokine genes, as well as cyclooxygenases and other genes involved in the inflammatory responses (57). The finding that TNF-α-triggers the rise in cytosolic calcium and cytokine production, via a novel pathway that uses the sequential activation of PLD1 and SphK, has profound implications for the development of strategies for therapeutic intervention against differential myeloid responses to inflammation.

Acknowledgment
We thank A.-K. Fraser-Andrews for proofreading the manuscript.

Disclosures
The authors have no financial conflict of interest.
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Letter of Retraction

I wish to retract the article “Phospholipase D1 Plays a Key Role in TNF-α Signaling” by Swaminathan Sethu, Grecia Mendez-Corao, and Alirio J. Melendez, The Journal of Immunology, 2008, 180: 6027–6034.

An investigation by the National University of Singapore concluded that in Fig. 1C, three cells in the 5 and 10 min points appear identical, but rearranged in location. The investigation also concluded that the cells in the 2, 5, and 10 min time points in Fig. 6B were identical to the 5, 15, and 30 min time points in Fig. 1A in Pushparaj et al. (The Journal of Immunology, 2009, 183: 221–227), which has been retracted.

The Investigative Committee of the National University of Singapore concluded that Dr. Melendez committed serious scientific misconduct. The committee found no evidence indicating that the coauthors were involved in the scientific misconduct.

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