Selective induction of phospholipase D1 in pathogen-activated human monocytes

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Phospholipase D (PLD) activation is part of the complex signalling cascade induced during phagocyte activation. Two PLD isoforms have been cloned, but their role in phagocyte functions is still poorly defined. We report that resting fresh circulating human monocytes expressed PLD1. PLD1 protein expression was rapidly down-regulated during cell culture. Lipopolysaccharide and pathogen-derived agonists (*Candida albicans*, arabinoside-terminated lipoarabinomannan and Gram-positive bacteria, but not mannose-capped lipoarabinomannan or double-stranded RNA) strongly induced PLD1 expression at both the mRNA and protein levels. Pro-inflammatory cytokines [interleukin (IL)-1 β and tumour necrosis factor α] had only a weak effect, whereas immune cytokines (IL-6 and interferon γ), anti-inflammatory cytokines (IL-13 and IL-10) and chemoattractants (fMet-Leu-Phe and macrophage chemoattractant protein 1) were

inactive. None of the agonists tested induced significant changes in the basal expression of PLD2 mRNA. Consistent with PLD1 up-regulation was the observation that PLD enzymic activity was higher in monocytes treated with active-pathogen-derived agonists than in control cells, when stimulated with PMA or with chemotactic agonists (fMet-Leu-Phe and C5a). Thus PLD2 seems to be a constitutive enzyme in circulating monocytes. Conversely, PLD1 is an inducible protein, rapidly regulated during culture conditions and selectively induced during cell activation. Therefore PLD1 might have a relevant role in immune responses against pathogens and in chronic inflammation.

Key words: chemotactic factors, innate immunity, signal transduction.

INTRODUCTION

Phagocytic cells are the first line of defence against intracellular pathogens [1]. Phagocyte activation in response to extracellular agonists is mediated by different classes of effector enzymes, including phospholipases [2]. Phospholipase D (PLD) hydrolyses phosphatidylcholine, producing phosphatidic acid and choline [2–4]. Phosphatidic acid can be metabolized further to bioactive lipids like diacylglycerol and lysophosphatidic acid, or it can be used for phospholipid biosynthesis [2,3,5]. Phosphatidic acid can also act as a second messenger, because a direct role in secretory vesicle budding [6] and in activation of the respiratory burst [5,7] has been reported. Moreover, the activation of phagocyte responses, including degranulation [8], phagocytosis [9] and the respiratory burst [7], has been related to increased PLD activity.

Two mammalian PLD isoforms, designated PLD1 and PLD2 (which exist in different splicing variants), have been cloned and characterized [10–13]. The two isoforms share some biochemical properties, such as positive regulation by PtdIns(4,5) P_2 and inhibition by oleate [5]. Nevertheless, the two forms are differentially regulated by protein kinase C and the small GTPases ARF and Rho [4,14].

Furthermore, PLD1 and PLD2 are only 50 % identical, localize to different intracellular compartments and display differences in terms of both basal activity and sensitivity to intracellular

activators and inhibitors [5,11]. These findings indicate that PLD isoforms are probably involved in as yet unknown different cellular functions [11].

Pro-inflammatory cytokines produced at the site of inflammation and immune reaction, and pathogens themselves, promote both the recruitment and the activation of phagocytic cells [15,16]. In phagocytes, both tyrosine-kinase-coupled [17] and G-protein-coupled [18–21] receptors induce a rapid (within minutes) induction of PLD activity. The activation of PLD activity by the CC chemokine RANTES was inhibited by Brefeldin A, a fungal metabolite that blocks the function of guanine nucleotide exchange proteins such as ARF [22]. fMet-Leu-Phe (fMLP), a prototypic bacterium-derived chemotactic agonist, was shown to induce the translocation of ARF-regulated PLD activity to the plasma membrane in human neutrophils [23]. It was reported recently that fresh circulating human monocytes express detectable levels of PLD1 protein [24].

The aim of the present study was to investigate the regulation of PLD1 and PLD2 expression in human monocytes exposed to pro-inflammatory and anti-inflammatory cytokines and to pathogen-derived agonists. In addition, PLD activity in resting and activated monocytes challenged with chemotactic agonists, as well as with PMA, a non-receptor-cell activator, was investigated in parallel studies. The results indicate that PLD1 is rapidly modulated by culture conditions and by a restricted set

Abbreviations used: Ara-LAM, arabinoside-terminated lipoarabinomannan; fMLP, fMet-Leu-Phe; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; Man-LAM, mannose-capped lipoarabinomannan; MCP, monocyte chemotactic protein; PLD, phospholipase D; PtdEt, phosphatidylethanol; TNF, tumour necrosis factor.

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of agonists at both the mRNA and protein levels. In contrast, PLD2 mRNA expression was not affected by the treatment conditions employed *in vitro*.

EXPERIMENTAL

Reagents

Tumour necrosis factor (TNF)- α and interleukin (IL)-1 β were from Sandoz (Basel, Switzerland), IL-13 was from Sanofi Elf Bio Recherches (Labege, France), interferon (IFN)- γ was from Roussel Uclaf (Romainville, France), IL-6 was from Serono (Genève, Switzerland) and monocyte chemotactic protein 1 (MCP-1) was from PeproTech (Rocky Hill, NJ, U.S.A.). IL-10 was a gift from Schering Plough (Kenilworth, NJ, U.S.A.). Lipopolysaccharide (LPS), poly(IC), dihydrocytochalasin B, fMLP, C5a and PMA were from Sigma (St Louis, MO, U.S.A.). Recombinant human PLD1 was prepared as described previously [12]. Mycobacteria-derived mannose-capped lipoarabinomannan (Man-LAM) and arabinoside-terminated lipoarabinomannan (Ara-LAM) were a gift from Dr J. T. Belisle (Colorado State University, Fort Collins, CO, U.S.A.). OK432, a freeze-dried preparation of *Streptococcus pyogenes*, was a gift from Chugai Pharmaceutical Co. (Tokyo, Japan). *Candida albicans* (A. T. C. C., Manassas, VA, U.S.A.) was grown and heat-inactivated as described previously [25]. All reagents not otherwise specified were from Sigma.

Cell preparation

Monocytes were separated from buffy coats from healthy blood donors as previously described [26], resuspended at 3×10^6 /ml in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (Hyclone, Logan, UT, U.S.A.), plated in hydrophobic Petriperm dishes (Heraeus, Vienna, Austria) and treated as specified in the text. Monocyte-derived macrophages were generated by incubating monocytes in Petriperm dishes in RPMI medium with 40% (v/v) autologous serum for 7 days [27].

Migration assay

Cell migration was evaluated with a chemotaxis microchamber technique. In brief, 27 μ l of chemoattractant or control medium [RPMI 1640 with 1% (v/v) fetal calf serum] were added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA, U.S.A.) [28]. A cell suspension (50 μ l; 1.5×10^6 cells/ml) was seeded in the upper chamber. The two compartments were separated by a polycarbonate filter (5 μ m pore size; Neuroprobe). The chamber was incubated at 37 °C in humidified air/CO₂ (19:1) atmosphere for 90 min. At the end of the incubation, filters were removed, stained and five high-power oil-immersion fields ($\times 100$) were counted.

Northern blot analysis and reverse transcription

Total RNA was extracted by the guanidium isothiocyanate method, processed, hybridized and blotted as described [29]. Human PLD1 and PLD2 probes were amplified from expressed sequence tag clones 283671 and 756888 (IMAGE Consortium, Research Genetics, Huntsville, AL, U.S.A.) respectively. The PLD1 probe corresponded to a specific fragment, absent from PLD2 mRNA (GenBank® accession number U38545; fragment 1589–2012), and did not distinguish between the PLD1a and PLD1b splicing variants. The PLD2 probe corresponded to a 650 bp fragment mainly located in the 3' untranslated region

with no significant similarity to the corresponding PLD1 region (GenBank® accession number AF033850; from position 2837 downstream). For reverse-transcriptase-mediated PCR, 1 μ g of total RNA was reverse-transcribed with reverse transcriptase from Moloney-murine-leukaemia virus (Life Technologies, Paisley, Renfrewshire, U.K.) and oligo(dT) and amplified by PCR with PLD1-specific oligonucleotides.

Western blot analysis

Monocytes treated with di-isopropylfluorophosphate (1 mM, 0 °C, 5 min; Fluka, Buchs, Switzerland) were resuspended in 100 μ l of lysis buffer [100 mM Tris/HCl (pH 7.4)/2 mM EDTA/100 mM NaCl/1% (v/v) Nonidet P40/0.1% SDS/2000 i.u./ml aprotinin/1 mM PMSF/100 μ M leupeptin/50 μ M pepstatin A/200 μ M NaF]. Proteins were separated by SDS/PAGE [8% (w/v) gel] and transferred to nitrocellulose paper (Bond-NC; Whatman, Maidstone, Kent, U.K.). Filters were incubated overnight at 4 °C with a polyclonal antiserum raised against the N-terminal portion of human PLD1 (1:200 dilution; Quality Controlled Biochemicals, Hopkinton, MA, U.S.A.). Immunoreactivity was detected by enhanced chemiluminescence (Amersham, Little Chalfont, Bucks., U.K.).

PLD activity determination

Monocytes (6×10^6 /ml) were labelled with 1 μ Ci/ml 1-*O*-[³H]octadecyl-*sn*-glycero-3-phosphocholine (Amersham) for 16 h. Monocytes (5×10^6 /ml in RPMI 1640 medium with 0.2% BSA) were prewarmed at 37 °C in the presence of 10 μ M dihydrocytochalasin B for 5 min, incubated with 2% (v/v) ethanol for 5 min and then stimulated with fMLP or C5a for 1 min, or with PMA for 15 min. Total lipids were extracted and separated by TLC as described previously [7]. [³H]-Phosphatidylethanol ([³H]PtdEt) was identified by co-migration with unlabelled standard (Biomol, Plymouth Meeting, PA, U.S.A.), recovered by scraping and quantified by liquid-scintillation counting. The radioactivity present in the [³H]PtdEt fraction was expressed as a percentage of the total radioactivity recovered from the lane.

FACS analysis

Cell staining was performed with mouse anti-(human CD88) (C5aR) (0.2 μ g/10⁶ cells; clone S5/1; Serotec, Oxford, U.K.) and its irrelevant control mouse antibody, IgG2a κ (UPC10; Sigma), followed by FITC-conjugated affinity-purified, isotype-specific goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) [30]. Binding of fMLP to monocytes was examined with FITC-labelled fMLP-Lys (FITC-fMLP) (Peninsula Laboratories, Belmont, CA, U.S.A.), as described previously [31]. Cells (10⁶) were incubated with FITC-fMLP (100 nM) for 30 min at 4 °C, washed and analysed by flow cytometry. To confirm specific binding, a 100-fold excess of unlabelled ligand was added to parallel samples. Analysis of fluorescence was performed by a FACStar Plus (Becton Dickinson, Mountain View, CA, U.S.A.) calibrated with Calibrite beads (Becton Dickinson).

Statistical analysis

Student's *t* test was used to analyse data for statistically significant differences.

RESULTS

Modulation of PLD1 expression in resting and LPS-treated human monocytes

In a first series of experiments, PLD activity was investigated after a prolonged activation (up to 48 h) with LPS, a prototypic activator of phagocytic cells [16,32]. Figure 1(A) shows that stimulation of monocytes by PMA in the presence of ethanol induced [3 H]PtdEt accumulation in both control and LPS-stimulated cells. PLD activity in fresh (overnight labelled) control monocytes was higher (1.8-fold; $P = 0.01$; Student's t test) than that observed in monocytes cultured *in vitro* for 48 h. However, culturing for 48 h in the presence of LPS (100 ng/ml) not only prevented the down-regulation observed in control cells, but resulted in a 3-fold increase in PMA-inducible PLD activity ($P = 0.0001$ for control compared with LPS-stimulated cells after 48 h in culture). Because human monocytes have been reported to express the PLD1 isoform [24], Western blot analysis was performed with a commercially available anti-PLD1 antibody. Figure 1(B) shows that fresh monocytes expressed an immunoreactive band that co-migrated with recombinant PLD1. PLD1 immunoreactivity was undetectable in control cells cultured *in vitro* for 48 h. In contrast, PLD1 immunoreactivity in LPS-treated cells was 2.5 ± 0.3 -fold higher than in fresh monocytes (mean $\pm$ S.D.; $n = 3$). Therefore the levels of PMA-inducible PLD activity are apparently correlated with the relative amount of PLD1 in the different experimental conditions.

PLD1 expression was also investigated by Northern blot

Figure 1 Modulation of PLD1 by culturing conditions and LPS stimulation

Monocytes were used after culture overnight (o/n) or after culture for 48 h in the absence or in the presence of 100 ng/ml LPS. (A) Monocytes (5×10^6 /ml), cultured and labelled as described in the Experimental section, were stimulated for 15 min with 100 nM PMA in the presence of 2% (v/v) ethanol. Lipid fractions were separated and the distribution of labelled lipids was evaluated by liquid-scintillation counting. Results are means $\pm$ S.D. for at least three independent experiments. Abbreviation: PET, PtdEt. (B) Western blot analysis of PLD1 protein expression. Proteins (700 μ g per lane) were separated on 8% (w/v) polyacrylamide gel, blotted, then incubated with a specific anti-PLD1 antiserum. Recombinant human PLD1 (std PLD1) is shown as reference. One of three similar experiments is shown. (C) Northern blot analysis of PLD1 mRNA expression; 10 μ g of total RNA was used in each lane. One of three similar experiments is shown. Staining of the gel with ethidium bromide is shown at the bottom. The positions of molecular-mass markers are indicated at the left.

Figure 2 Time course of expression of PLD1 mRNA and protein in LPS-activated human monocytes

Monocytes were incubated at 37 °C for a total of 48 h; LPS (100 ng/ml) was added at different times during the incubation. (A) Northern blot analysis of PLD1 mRNA expression; 10 μ g of total RNA was used in each lane. One of three similar experiments is shown. Staining of the gel with ethidium bromide is shown at the bottom. (B) Western blot analysis of PLD1 protein expression. Proteins (700 μ g per lane) were separated on 8% (w/v) polyacrylamide gel, blotted, then incubated with a specific anti-PLD1 antiserum. Recombinant human PLD1 (std PLD1) is shown as reference. One experiment representative of three independent experiments is shown. The positions of molecular-mass markers are indicated at the left.

analysis. Two specific PLD1 transcripts of approx. 6.5 kb and approx. 4.5 kb [33] were detected in LPS-treated monocytes (Figure 1C). In contrast, in untreated cells PLD1 mRNA could be observed only after prolonged (more than 6 days) exposure of the film or by reverse-transcriptase-mediated PCR performed with PLD1-specific primers (results not shown). With the same molecular probe, PLD1 mRNA was readily detectable by Northern blotting in HL60 total RNA (results not shown), as reported previously [10,34]. These results suggest that PLD1 transcription is very low in resting circulating monocytes and that PLD1 expression is strongly up-regulated by LPS. We also investigated PLD2 expression. Northern blot analysis revealed that PLD2 mRNA was abundantly expressed and readily detectable (after only 8 h of film exposure) in both resting and LPS-activated cells, with no apparent effect of treatment (see below). Unfortunately, the analysis of PLD2 protein was hampered by a lack of commercially available specific antibodies.

The kinetics of LPS-induced PLD1 up-regulation was investigated in greater detail. Figure 2(A) shows that the effect of LPS on mRNA expression was detectable after only 8 h of stimulation and was maximal at 24 h. Thereafter, PLD1 expression decreased. At these later time points, LPS induces IL-10 production that acts as a negative feedback mechanism [35]. To investigate whether the LPS-induced up-regulation of PLD1 expression was sensitive to IL-10 action, monocytes were incubated with LPS for 24 h in presence and in the absence of 10 ng/ml IL-10. In the presence of IL-10, LPS-induced PLD1 expression was inhibited by 73% ($n = 2$; results not shown). A similar degree of inhibition was observed when IL-1 β expression was investigated under the same experimental conditions (results not shown). The kinetics of PLD1 protein expression in LPS-treated monocytes was delayed only slightly compared with that observed by Northern blotting. LPS effect was time-dependent, with PLD1 protein being barely detectable after 8 h of treatment with LPS and reaching a plateau after 24–48 h of stimulation (Figure 2B).

Figure 3 Northern-blot analysis of PLD1 and PLD2 expression in human monocytes and monocyte-derived macrophages (Mφ)

Cells were incubated in the presence of different agonists for 24 h. Total RNA was extracted and blotted as described in the Experimental section; 10 µg of total RNA was used in each lane. Monocytes were stimulated (**A**) with 100 ng/ml LPS, 50 ng/ml OK432, 100 µg/ml *C. albicans*, 1 µg/ml Ara-LAM, 3 µg/ml Man-LAM, 100 µg/ml poly(IC) or (**B**) with 500 i.u./ml IFN-γ, 20 ng/ml IL-1β, 500 i.u./ml TNF-α, 20 ng/ml IL-13, 10 ng/ml IL-6. (**C**) Monocytes and monocyte-derived macrophages were incubated with 100 ng/ml LPS. Overnight exposures of one experiment representative of two to six independent experiments are shown. Staining with ethidium bromide is shown at the bottom. The positions of molecular-mass markers are indicated at the left.

Selective up-regulation of PLD1 by pathogens

After these observations we performed a more extensive analysis of the expression profiles of PLD1 and PLD2 in activated monocytes. As shown in Figure 3(A), exposure to *C. albicans* (yeast) for 24 h was as effective as LPS (derived from Gram-negative bacteria) in inducing PLD1. Under the same experimental conditions, Ara-LAM (mycobacteria-derived) and Gram-positive bacteria (OK432) were far less active ($18 \pm 8\%$ and $14 \pm 7\%$ of the LPS effect respectively; $n = 3$). Conversely, poly(IC) (double-stranded RNA) and Man-LAM (mycobacteria-derived) were completely inactive (Figure 3A). None of the agonists tested influenced the basal expression of PLD2, with only modest effects of LPS (2.6 ± 1.2 -fold control values; $n = 6$) and Ara-LAM (2.2 ± 1.2 -fold control values; $n = 3$) that did not reach statistical significance.

Monocyte activation typically leads to the induction of pro-inflammatory cytokines that modulate the immune response to pathogens [36]. Figure 3(B) shows that IL-1β, TNF-α, IL-6 and IFN-γ, used at their optimal concentrations for 24 h, did not mimic the effect of LPS or *C. albicans* on PLD1 expression. IL-1β and TNF-α were only weak PLD1 inducers ($11 \pm 3\%$ and $10 \pm 1\%$ of the LPS effect respectively; $n = 3$) and the two cytokines added together were, at best, only additive. Complete inactivity was shown by IL-6 and IFN-γ (both in the absence and in the presence of LPS), as well as IL-13 and IL-10, two prototypical anti-inflammatory cytokines, and two chemotactic attractants (fMLP and MCP-1) (Figure 3B, and results not

shown). Finally, the expression of PLD1 and PLD2 in monocyte-derived macrophages was investigated. Similarly to monocytes, resting and LPS-stimulated monocyte-derived macrophages expressed PLD2 mRNA, whereas PLD1 expression was detectable only in LPS-stimulated cells (Figure 3C).

To investigate the biological relevance of the induction of PLD1 mRNA, PLD enzymic activity was evaluated. Figure 4 shows that PLD activity was increased in cells treated with LPS and *C. albicans* to similar extents. The increase was detectable along the whole PMA dose-response curve tested; at the optimal PMA concentration of 100 nM it was 4.6 ± 2.9 -fold ($n = 8$; $P < 0.01$) and 3.3 ± 1.8 -fold ($n = 3$; $P < 0.01$) the control values for LPS and *C. albicans* respectively. Consistent with the reported effect at mRNA level was the observation that Ara-LAM and Gram-positive bacteria (OK432) were far less active (1.4 ± 0.3 -fold and 1.3 ± 0.1 -fold increase over control, $n = 3$; $P < 0.05$), whereas Man-LAM was completely inactive (Figure 4C).

Up-regulation of PLD activity by chemotactic agonists in monocytes cultured with LPS

Localization of monocytes to the sites of inflammation and immune reaction is directed by chemotactic factors [15]. Engagement of chemotactic receptors results in PLD activation [4,5,7,8,18,19,21–23]. PLD activity was investigated in monocytes cultured for 48 h in the presence of LPS. fMLP and C5a were selected as chemotactic agonists because the membrane ex-

Figure 4 PMA-stimulated PLD activity in resting and pathogen-activated human monocytes

Monocytes ($10^7/\text{ml}$), cultured and labelled as described in the Experimental section, were stimulated for 15 min with different PMA concentrations (**A**, **B**) or with 100 nM PMA (**C**) in the presence of 2% (v/v) ethanol. Lipid fractions were separated and the distribution of labelled lipids was evaluated by liquid-scintillation counting. Results are means $\pm$ S.D. for eight (**A**) and three (**B**, **C**) independent experiments. * $P < 0.05$, ** $P < 0.01$ by paired t test compared with the control group. Abbreviation: PEt, PtdEt.

pression of their receptors was not changed after LPS treatment (Figures 5A and 5B), nor were monocytes able to migrate to fMLP (Figure 5C). Figure 5(D) shows that both fMLP ($1 \mu\text{M}$) and C5a (50 ng/ml) induced [^3H]PtdEt accumulation in monocytes and that their effect was greater in LPS-treated monocytes than in cultured control cells (respectively 2.2-fold and 2.7-fold the control).

DISCUSSION

The present study shows that in circulating human monocytes PLD1 expression is rapidly modulated during culture *in vitro* and by pro-inflammatory agonists. PLD1 protein, detectable in fresh

circulating cells, is rapidly down-regulated by culture overnight. Incubation in the presence of pathogen-derived agonists not only prevents the down-regulation of PLD1, but also strongly increases its expression at both the mRNA and protein levels. Accordingly, monocyte-derived macrophages do not express detectable levels of both PLD1 mRNA and protein *in vitro*. These results suggest that PLD1 might not be the most relevant isoform in resting-tissue macrophages *in vivo*. Conversely, PLD2 expression was detectable by Northern blot analysis in both fresh monocytes and monocytes cultured *in vitro*. Interestingly, PLD2 expression was not modulated during cell activation. In a recent study [24], PLD activity present in fresh (labelled for 90 min) circulating monocytes was ascribed to PLD1 expression. In the same study, PLD2 protein could not be detected by immunoprecipitation experiments with a PLD1/PLD2 non-specific antibody. Unfortunately, in the present study, we have been unable to investigate the expression of PLD2 protein owing to the lack of commercially available antibodies. It is therefore not known whether PLD2 is also expressed at the protein level in cultured monocytes. However, on the basis of the results presented here, we postulate that low levels of PLD2 expression are likely to be responsible for the PLD enzymic activity present in resting cultured monocytes (this study) and in monocyte-derived macrophages [37].

The interaction of phagocytes with pathogens or pathogen-induced soluble mediators promotes cell activation leading to the onset of inflammatory reactions [16,38]. We report here that during this process PLD1 expression is strongly up-regulated at both mRNA and protein levels. This event presented two levels of specificity. First, the expression of PLD1, but not that of PLD2, was specifically induced during monocyte and monocyte-derived macrophage activation. Secondly, among a large series of monocyte activators tested, only a subgroup of pathogen-derived agonists (i.e. LPS, *C. albicans*, OK432 and Ara-LAM) provided a strong signal for PLD1 induction. Moreover, the ineffectiveness of pro-inflammatory cytokines in mimicking pathogen-derived mediators suggested a direct effect of these cell activators on PLD1 induction.

It is interesting to note that most of the active agonists are known to interact with receptors that belong to the Toll-like receptor (TLR) family, a group of membrane proteins with an intracellular domain highly similar to the IL-1 receptor [32,39]. It was recently shown that LPS signals through the interaction with TLR4 [40], whereas TLR2 is the receptor for Gram-positive bacteria, lipoarabinomannans and fungi [32,39]. In contrast, the inactive agonists, such as IL- 1β , TNF- α , IFN- γ and poly(IC), do not use this family of receptors. The signalling cascade used by Tolls and IL-1 receptor are similar to a large extent, but some differences have been reported [41]. The observation that major (LPS) and weak (IL- 1β) PLD1 inducers use receptors with similar but distinct signalling properties might lead to the identification of specific transduction pathways regulating PLD1 expression.

Previous reports described the regulation of expression of PLD isoforms during cell differentiation [33,34]. In the present study we describe the selective induction of PLD1 during monocyte activation. This situation is reminiscent of other inflammatory enzymic systems in phagocytes also characterized by constitutive and inducible enzyme isoforms, namely cyclooxygenase and nitric oxide synthase [42]. Taken together, these observations propose a role for PLD1 in the late-phase inflammatory response, including macrophage responses in the context of chronic inflammation. In this regard, it is of particular interest that PLD activity is strictly required for the phagocytosis and killing of *Mycobacterium tuberculosis* by macrophages [9,37] and that up-regulation of PLD1 was induced by Ara-LAM, a *M.*

Figure 5 Chemotactic agonist-stimulated PLD activity in resting and LPS-activated human monocytes

Human monocytes were incubated with 100 ng/ml LPS for 48 h. **(A, B)** Flow cytometry analysis of fMLP receptor **(A)** and C5a receptor **(B)** in monocytes incubated in the absence (thin lines) or in the presence of LPS (thick lines) for 48 h. Dotted lines represent the reactivity of isotype-matched control monoclonal antibodies. Results of a single experiment, representative of at least four, are shown. Percentages (%) of positive cells and mean channel fluorescence (mean) are reported. **(C)** The migration of monocytes in response to fMLP was evaluated in a 48-well Boyden chamber. Results of one experiment performed in triplicate are shown and are representative of two independent experiments. **(D)** Monocytes were stimulated for 15 min with 1 μ M fMLP or 50 ng/ml C5a in the presence of 2% (v/v) ethanol. Lipid fractions were separated and the distribution of labelled lipids was evaluated by liquid-scintillation counting. Results are means $\pm$ S.D. for three independent experiments. Abbreviation: PET, PtdEt.

tuberculosis component. It is therefore conceivable that both PLD1 and PLD2 participate in the cytotoxic response against *M. tuberculosis* in infected macrophages. In this context, it is also interesting to note that IL-10 inhibits LPS-induced PLD1 activation, indicating a counteracting effect of anti-inflammatory mediators on pathogen-mediated PLD1 induction.

The modulation of PLD1 expression observed in different culture conditions was of biological relevance. PLD activity in response to PMA activation paralleled the results obtained by Northern blot and Western blot analyses. A decrease in enzymic activity was observed in cells incubated for 48 h *in vitro* compared with cells labelled overnight; an increase in PLD activity was observed in cells activated by pathogen-derived agonists. Stimulated monocytes also showed an increase in PLD activity after chemotactic receptor activation. For this analysis, C5a (a component of the complement activation cascade) and fMLP (a formylated peptide present on bacterial membranes) were selected because the expression of their receptors was unaffected by stimulation with LPS. Exposure of LPS-treated cells to optimal concentrations of C5a and fMLP resulted in a greater PLD activity than that observed in control monocytes. Because the chemotactic responses of control and LPS-treated cells were similar, it is likely that the increased PLD activity does not reflect a general priming effect by LPS. Therefore, although a

role for PLD2 cannot be excluded, these results strongly suggest that PLD1 is coupled to chemotactic receptor activation. An ARF-regulated PLD activity was translocated to the plasma membrane in neutrophils stimulated with fMLP [23]. A Rho-dependent PLD activity was implicated in stress fibre formation induced by the activation of lysophosphatidic acid receptor, a serpentine receptor. Finally, PLD1 was found to be localized to the cytoskeletal fraction in HL-60 cells [43,44]. Taken together, these results suggest that PLD1 might be involved in the reorganization of the cytoskeleton that follows the activation of chemotactic receptors.

In summary, this study shows that PLD1 and PLD2 are differentially expressed and regulated in resting and activated human monocytes. PLD1 expression is rapidly modulated in culture conditions and during cell activation. The regulation observed at the mRNA level was correlated with protein expression and PLD activity. The results presented in this study therefore suggest different roles for the two PLD isoforms in mononuclear phagocytes, with PLD2 having a housekeeping function and PLD1 being rapidly modulated during phagocyte activation. Finally, the selective induction of PLD1 by pathogens suggests a possible role for this isoform in the late phase of the immune response (e.g. T helper cell type 1 response to *M. tuberculosis*) and in chronic inflammation.

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