PLD1 rather than PLD2 regulates phorbol-ester-, adhesion-dependent and Fcγ-receptor-stimulated ROS production in neutrophils

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Summary

The signalling lipid phosphatidic acid (PA) is generated by the hydrolysis of phosphatidylcholine (PC), which is catalysed by phospholipase D (PLD) enzymes. Neutrophils, important cells of the innate immune system, maintain the body's defence against infection. Previous studies have implicated PLD-generated PA in neutrophil function; these have relied heavily on the use of primary alcohols to act as inhibitors of PA production. The recent development of isoform-selective small molecule inhibitors and the generation of a knockout mouse model provide us with accurate tools to study the role of PLDs in neutrophil responses. We show that PLD1 is a regulator of phorbol-ester-, chemoattractant, adhesion-dependent and Fcγ-receptor-stimulated production of reactive oxygen species (ROS) in neutrophils. Significantly we found that this role of PLD is isoform specific: the absence of PLD2 does not negatively affect these processes. Contrary to expectation, other functions required for an efficient immune response operate effectively in Pld2-deficient neutrophils or when both isoforms are inhibited pharmacologically. We conclude that although PLD1 does have important regulatory roles in neutrophils, the field has been confused by the use of primary alcohols; now that gold standard Pld-knockout mouse models are available, previous work might need to be reassessed.

Key words: Neutrophil, Phospholipase D, Reactive oxygen species

Introduction

Phospholipase D (PLD) enzymes catalyse the hydrolysis of phosphatidylcholine (PC) to generate the signalling lipid phosphatidic acid (PA). Activity of PLD and its product, PA, has been implicated in regulating a wide range of cellular responses, including neutrophil functions important for an effective mammalian immune system. As a major part of the body's anti-bacterial and fungal defence machinery, neutrophils are required to carry out a range of roles; guided by inflammatory mediators, they migrate from the bloodstream to sites of infection where foreign cells are engulfed and degraded by the release of reactive oxygen species (ROS) and proteolytic granules. A number of key neutrophil functions, such as migration, phagocytosis, actin cytoskeleton remodelling, degranulation and respiratory burst activation have been associated with PLD activity and PA (Agwu et al., 1991; Bauldry et al., 1991; Zhou et al., 1993; Serrander et al., 1996; Cadwallader et al., 2004; Levy et al., 2005; Powner et al., 2005; Zouwail et al., 2005; Corrotte et al., 2006; Iyer et al., 2006; Lehman et al., 2006; Carrigan et al., 2007; Powner et al., 2007; Chae et al., 2008; Nishikimi et al., 2009; Su et al., 2009).

Many of the previous studies that established a role for PLD-generated PA in neutrophil function relied heavily on the use of primary alcohols to act as inhibitors of PA production, by taking advantage of their ability to act as better nucleophilic acceptors in a transphosphatidylation reaction in which phosphatidyl–PLD intermediates normally use water in the formation of PA. However more recent work has begun to raise concerns over potential off-target effects or a lack of complete inhibition of PA production when using primary alcohols, such as butan-1-ol, to inhibit PLD, even when the tertiary alcohol has been used as a control (Skippen et al., 2002; Huang et al., 2005; Huang and Frohman, 2007; Su et al., 2009; Yanase et al., 2010). Work has also been carried out using PLD-isoform-selective siRNAs to inhibit PLD activity, which is a more lengthy approach, with effects taking place over hours or days and in many cases the knockdown is incomplete. This increases the possibility of secondary effects on other signalling pathways, whereas utilising short-acting PLD-isoform-selective inhibitors or long-term transgenic mouse models shown not to express PLD remove this potential problem.

Recently, the first report of a Pld1-knockout mouse model has been described (Elvers et al., 2010). The authors study the function of PLD in the process of platelet activation and aggregation and find that platelets lacking PLD1 activity displayed impaired integrin activation under high shear conditions (Elvers et al., 2010). The role of PLD1 in neutrophil function has yet to be studied in transgenic mice that no longer express PLD1, or through the use of efficient isoform-selective inhibitors.

In this study, we have taken advantage of the recent development and characterisation of PLD-isoform-selective small molecule inhibitors (Scott et al., 2009) and the generation of transgenic mice that do not express Pld2 to provide us with more precise tools to study the role of PLDs in neutrophil responses. Our data show that PLD1 activity is required for neutrophils to produce ROS, a process that is vital for the removal of infective agents, in response to: (1) the soluble stimuli N-formyl methionyl leucyl phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA); (2) adhesion to integrin ligands; and (3) stimulation of Fcγ immune receptors. Interestingly, we found that the absence of PLD2 does not negatively affect these processes. Furthermore, many other functions required for an efficient immune response such as chemotaxis, chemokinesis and adhesion to integrin ligands can still operate effectively in Pld2-knockout neutrophils, or when both PLD1 and PLD2 are inhibited pharmacologically. These data indicate an important role for PLD1 in the immune response and they provide clear evidence for exclusive roles of the PLD isoforms in neutrophils. This work, using a knockout mouse model, provides some clarity to the body of previous literature, which established a role for PLD and PA in neutrophil functions using the non-selective primary alcohol butan-1-ol as a PLD inhibitor.

Results

Production of Pld2-knockout mouse model and characterisation of isoform-selective PLD inhibitors

To analyse the function of PLD in neutrophils, two alternative approaches were adopted: a genomic approach and a pharmacological method. We disrupted the *Pld2* gene in mice by standard gene-targeting methods (supplementary material Fig. S1). Pld2-knockout (Pld2KO) mice were viable, born in expected mendelian ratios, developed normally, were fertile and did not display any behaviour distinguishable from wild-type (WT) litter mates. Western blot analysis using a rat polyclonal antibody generated against the C-terminus of mouse PLD2 (see the Materials and Methods) confirmed the absence of PLD2, indicating the successful inactivation of the *Pld2* gene in the Pld2KO mice (Fig. 1Ai). PLD1 protein levels were also analysed by western blot in neutrophils from WT and Pld2KO mice, and no differences were observed (Fig. 1Aii) indicating that compensation has not occurred and that PLD1 is not more highly expressed in the Pld2KO. Normal bone-marrow-derived neutrophil numbers and purities were obtained (further characterisation of the Pld2KO mice will be published elsewhere).

To analyse fully the roles of both PLD1 and PLD2 in neutrophil function, we used a new generation of isoform-selective inhibitors developed by Scott and colleagues (Scott et al., 2009): VU0155069 (PLD1-selective) and VU0155056 (PLD1/2 dual). We assessed these compounds in freshly isolated bone-marrow-derived neutrophils and determined that they functioned efficiently as PLD inhibitors. Neutrophil PA production was measured by mass spectrometry. PMA stimulation led to a threefold increase in PA (Fig. 1B). This increase was abolished in the presence of a maximal concentration of the PLD1 and PLD2 (PLD1/2) dual inhibitor, restoring PA production to basal levels (Fig. 1B). Inhibitory dose curves in cell-based superoxide assays were

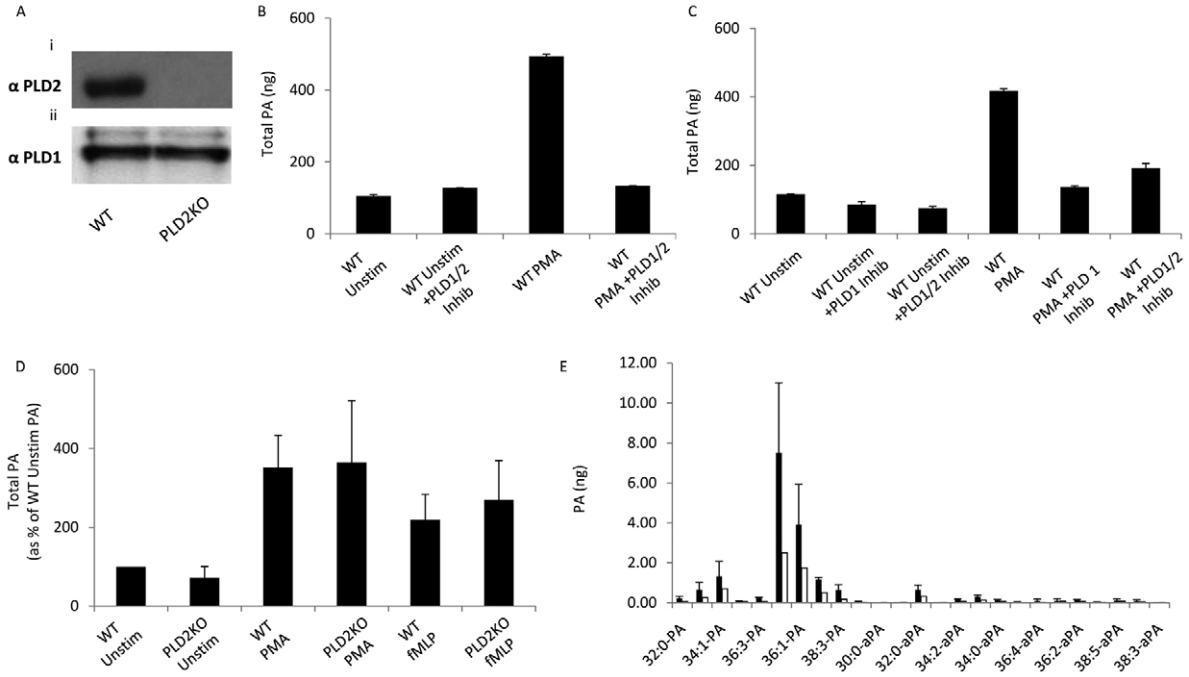

Fig. 1. Characterisation of PLD2KO mouse model and isoform-selective PLD inhibitors. (**A**) Western blot analysis using (i) a monoclonal antibody generated against the C-terminus of mouse PLD2 (MAC444) confirming the absence of PLD2, and (ii) a polyclonal PLD1 antibody (Cell Signalling) confirming no change in PLD1 protein levels in the Pld2KO. (**B**,**C**) Total PA was analysed by LCMS in WT mouse neutrophils incubated with or without PLD1/2 dual inhibitor (10 μM; 10 minutes) (B); PLD1/2 dual inhibitor (500 nM) or PLD1 Inhibitor (1 μM) (C) and stimulated with or without PMA (100 nM; 10 minutes). Data shown are from a representative experiment; data points were measured in duplicate. (**D**) Total PA was analysed by LCMS in WT and Pld2KO mouse neutrophils stimulated with or without PMA (100 nM; 10 minutes) or fMLP (1 μM; 5 minutes). Data are expressed as a percentage of WT unstimulated total PA and are accumulated from three experiments where each data point was performed in duplicate. WT vs Pld2KO shows no significant difference: unstimulated P=0.231; PMA P=0.932; fMLP P=0.319. (**E**) Individual PA species were analysed by LCMS in WT (black) and Pld2KO (white) mouse neutrophils without stimulation.

created to ensure inhibitors were used at a relevant inhibitory concentration (IC_{50}) in subsequent neutrophil assays (data not shown). PMA-stimulated PA production was also measured in neutrophils after pre-incubation with the isoform-discriminating inhibitors at these more selective concentrations (IC_{50}s); PA levels were found to be 55–60% of that of the control cells (Fig. 1C) (these concentrations were then used in subsequent assays). Comparison of PA generation between WT and Pld2KO neutrophils demonstrated a small reduction in basal PA, but no reduction in either PMA- or fMLP-stimulated generation (Fig. 1D). A lack of any significant increases in PA production in the Pld2KO (Fig. 1D) could also indicate that PLD1 has not overcompensated for the lack of PLD2 by producing further PA. The reduction in basal PA in the neutrophils was mostly in 36:1 PA (Fig. 1E), this is a species we have previously demonstrated to be, at least partly, produced through PLD activity (Pettitt et al., 2001); this adds evidence to the proposition that distinct species of PA act as selective signalling molecules.

PLD1 is a regulator of phorbol-ester- and chemoattractant-induced ROS production

Neutrophils were assessed for their ability to produce ROS, a process that is vital for eliminating bacterial and fungal pathogens from the body. ROS production was measured using a horseradish peroxidase (HRP)-dependent chemiluminescence assay. In all assays, neutrophils were primed with TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF) before stimulation; where experiments were performed on unprimed cells, the same effects were obtained. Expression of the cytoplasmic components of the NADPH oxidase ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) was found to be normal by western blot analysis in Pld2KO neutrophils (supplementary material Fig. S2A).

Pre-incubation with the PLD1/2 dual inhibitor led to an enhanced ROS response to the formylated bacterial peptide fMLP, but a diminished PMA-induced response; however, comparison of WT and Pld2KO cells in these assays showed little difference in the total production of ROS under these conditions (Fig. 2). Further clarity was obtained by using the PLD1-selective compound, which still produced a significantly elevated fMLP response and abrogated the PMA response (Fig. 2). There was no difference in the kinetics of fMLP-stimulated ROS production but only in the total amount produced. Pre-incubation with the selective inhibitors upon PMA stimulation revealed a slower rate of ROS production, as well as an overall reduction in the response (Fig. 2). Some experiments were performed using maximal concentrations of inhibitors; similar, but enhanced, effects were observed (data not shown). At these maximal inhibitor concentrations, PMA-stimulated ROS generation was essentially fully inhibited.

These data provide a novel indication of a distinction between the roles of the two PLD isoforms in ROS production in neutrophils. In the absence of PLD2, neutrophils remain capable of producing ROS, suggesting that PLD2-generated PA is not required for NADPH oxidase activation in response to fMLP- and PMA-induced signalling. The isoform-selective distinction is common to stimulation with both fMLP, a GPCR agonist, and PMA, a non-physiological direct activator of PKCs; however, the effect of

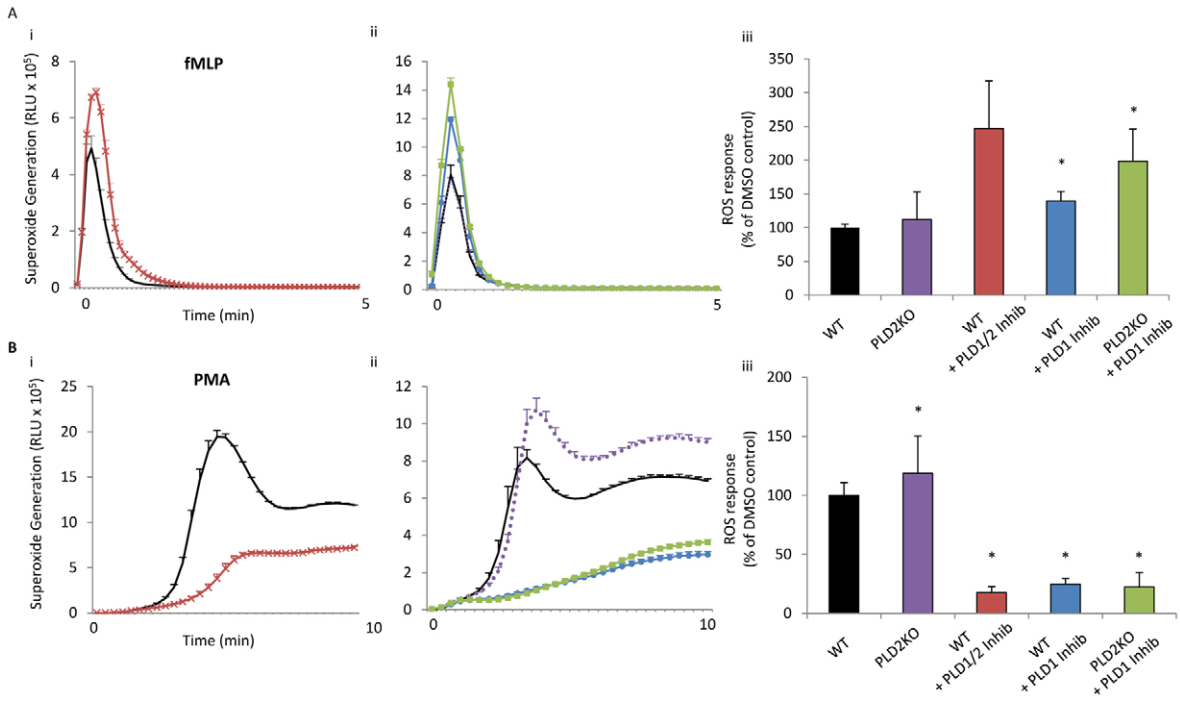

Fig. 2. PLD1 regulation of neutrophil ROS generation in response to soluble stimuli fMLP and PMA. (**A**,**B**) Representative time course (i,ii) and total ROS generated (iii) from WT (black in i–iii) and Pld2KO (purple in ii and iii) neutrophils incubated with or without PLD1/2 dual inhibitor (500 nM) (red in i and iii) or PLD1 inhibitor (1 µM) (WT blue in ii and iii; Pld2KO green in ii and iii) under fMLP (1 µM; 3 minutes) (A) or PMA (100 nM; 10 minutes) (B) stimulation. Results (iii) are expressed as a percentage of individual WT controls over 3 minutes (fMLP) or 10 minutes (PMA) and are collated from at least three independent experiments (WT vs Pld2KO, $n \geq 18$). fMLP: Pld2KO, $P=0.291$; WT + PLD1/2 Inhib, $P=0.072$; WT + PLD1 Inhib, $P=0.012$; Pld2KO + PLD1 Inhib, $^*P=0.001$. PMA: Pld2KO, $P=0.034$; WT + PLD1/2 Inhib, WT + PLD1 Inhib and Pld2KO + PLD1 Inhib, all $^*P<0.0001$.

PLD1 inhibition is different: ROS production is enhanced under fMLP stimulation and reduced with PMA. The differential effects of the two stimuli are intriguing and not yet fully understood. PLD appears not to be essential for ROS production downstream of the GPCR agonist fMLP, which is primarily regulated through PI3K activity; in fact, removing PLD activity appears to enhance ROS production in this setting. This could suggest a possible suppressive or regulatory role for PLD1 in the signalling pathway. By contrast, PLD activity does appear to be required for maximal ROS production downstream of the PKC activator, PMA, indicating a role for PLD in signalling to the NADPH oxidase downstream of PMA.

PLD1 is a regulator of adhesion-induced ROS production

Upon adhesion to extracellular matrices, neutrophils produce ROS. Incubation of WT neutrophils with the PLD1/2 dual inhibitor led to a significant decrease in total ROS production upon adhesion to a fibrinogen and a polyvalent integrin ligand surface (pRGD) matrix (66% and 55% of WT response, respectively) (Fig. 3A,B). Further elucidation of the response using neutrophils from the bone marrow of Pld2KO mice and the PLD1-preferring compound showed again that the effect was due to the reduction in PLD1, but not PLD2 activity (Fig. 3). There was no difference in the kinetics of integrin-dependent ROS production in the presence of the PLD-selective inhibitors, or in neutrophils isolated from PLD2KO mice, but only in the total amount produced (Fig. 3A,Bi,Bii).

Treatment of WT and Pld2KO neutrophils with PLD-selective inhibitors induced no defect in adhesion or spreading upon fibrinogen and pRGD matrices (Fig. 3C). Therefore the PLD1-dependent component of ROS production upon adhesion to integrin ligands is not due to a defect in the ability of cells to adhere and/or spread, but rather in a signalling pathway between integrin binding and NADPH oxidase activation.

PLD1 is a regulator of Fcγ-receptor-induced ROS production

Mouse neutrophils recognise antibody-opsonised particles through Fcγ receptors (FcγRIII and FcγRIV) (Jakus et al., 2008;

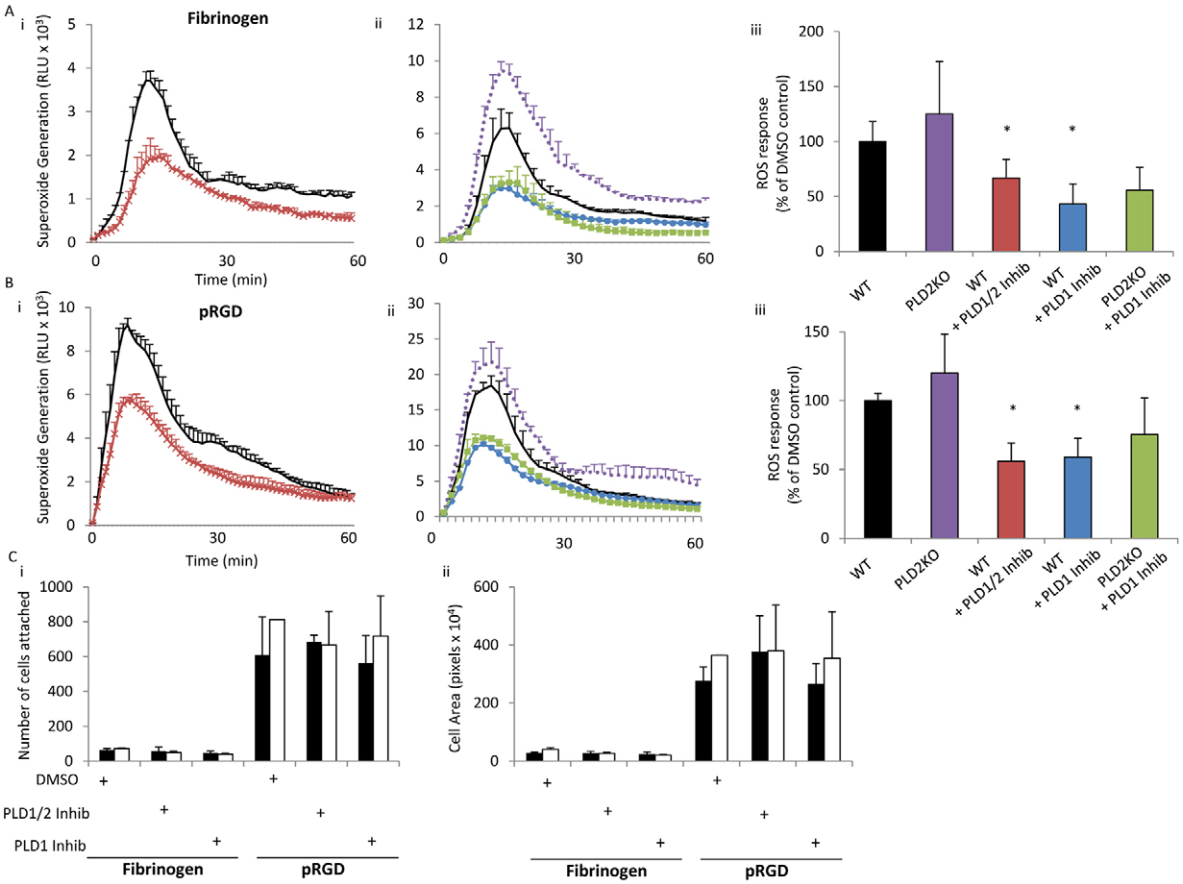

Fig. 3. PLD1 regulation of neutrophil ROS generation in response to adhesion. (**A**,**B**) Representative time course (i and ii) and total ROS generated (iii) from WT (black in i, ii, iii) and Pld2KO (purple in ii and iii) neutrophils incubated with or without PLD1/2 dual inhibitor (500 nM) (WT red in i and iii) or PLD1 inhibitor (1 µM) (WT blue in ii and iii; Pld2KO green in ii and iii) upon adhesion to fibrinogen (150 µg/ml) (A) and pRGD-coated (20 µg/ml) surfaces (B) (1 hour) with addition of (TNFα 20 ng/ml). Results (iii) are expressed as a percentage of individual WT controls over 60 minutes and were collated from at least three independent experiments. Fibrinogen: Pld2KO, P=0.263; WT + PLD1/2 Inhib, *P=0.039; WT + PLD1 Inhib, *P=0.033; Pld2KO + PLD1 Inhib, P=0.137. pRGD: Pld2KO, P=0.119; WT + PLD1/2 Inhib, *P=0.024, WT + PLD1 Inhib, *P=0.044, and Pld2KO + PLD1 Inhib, P=0.297. (**C**) Adhesion (i) (number of cells attached) and spreading (ii) (total number of pixels total cell area covers) of WT (black) and Pld2KO (white) neutrophils incubated with or without PLD inhibitors upon fibrinogen (150 µg/ml) and pRGD (20 µg/ml) surfaces (1 hour) with addition of (TNFα 20 ng/ml). Results are from at least 24 fields of view from at least two independent experiments.

Nimmerjahn and Ravetch, 2008). Stimulation by IgG-opsonised sheep red blood cells (SRBCs) elicits the production of ROS (Fig. 4A). In the presence of the PLD1/2 dual inhibitor, the total amount of ROS produced was reduced (65% of the WT response) (Fig. 4A), whereas the phagocytosis of IgG-SRBC was not affected (data not shown). Pre-incubation with the PLD1-selective compound led to a similar inhibition, whereas the total amount of ROS produced in Pld2KO neutrophils was not significantly different from that in the WT.

Neutrophil activation upon an immobilised IgG immune complex also signals through Fcγ receptors (in the absence of phagocytosis) and stimulates a ROS response by mimicking cell activation upon immune complex deposition caused by the transfer of antibodies to self-antigens in autoimmune diseases (Jakus et al., 2008). Inhibition of PLD1 activity led to a significant reduction in ROS production upon an anti-BSA immune complex (35% of WT); however, Pld2KO neutrophils showed no defect in total ROS produced (Fig. 4B). Hence PLD1, but not PLD2, is involved in Fcγ-receptor-mediated neutrophil responses.

PLD is not a regulator of ROS production induced by complement receptor signalling

Inhibition of PLD activity had no effect on ROS production stimulated by serum-opsonised *S. aureus* particles (Fig. 4C). It has been previously shown by Anderson and colleagues (Anderson et al., 2008) that *S. aureus*-induced ROS responses are independent of Fcγ receptor signalling, but are dependent upon complement receptor signalling. Hence, no role for PLD in signalling downstream of the complement receptor was discovered.

Neutrophil chemokinesis, chemotaxis and adhesion can occur in the absence of PLD activity

Some of our results were unexpected in the context of much of the literature on the subject. Therefore, it was important to further investigate the role of PLD in other established neutrophil cell-based assays using the new generation PLD inhibitors and Pld2KO neutrophils. Neutrophil chemokinesis-stimulated non-directional migration was assessed by bath application of fMLP. WT cells treated with both vehicle control and PLD1/2 dual inhibitor were

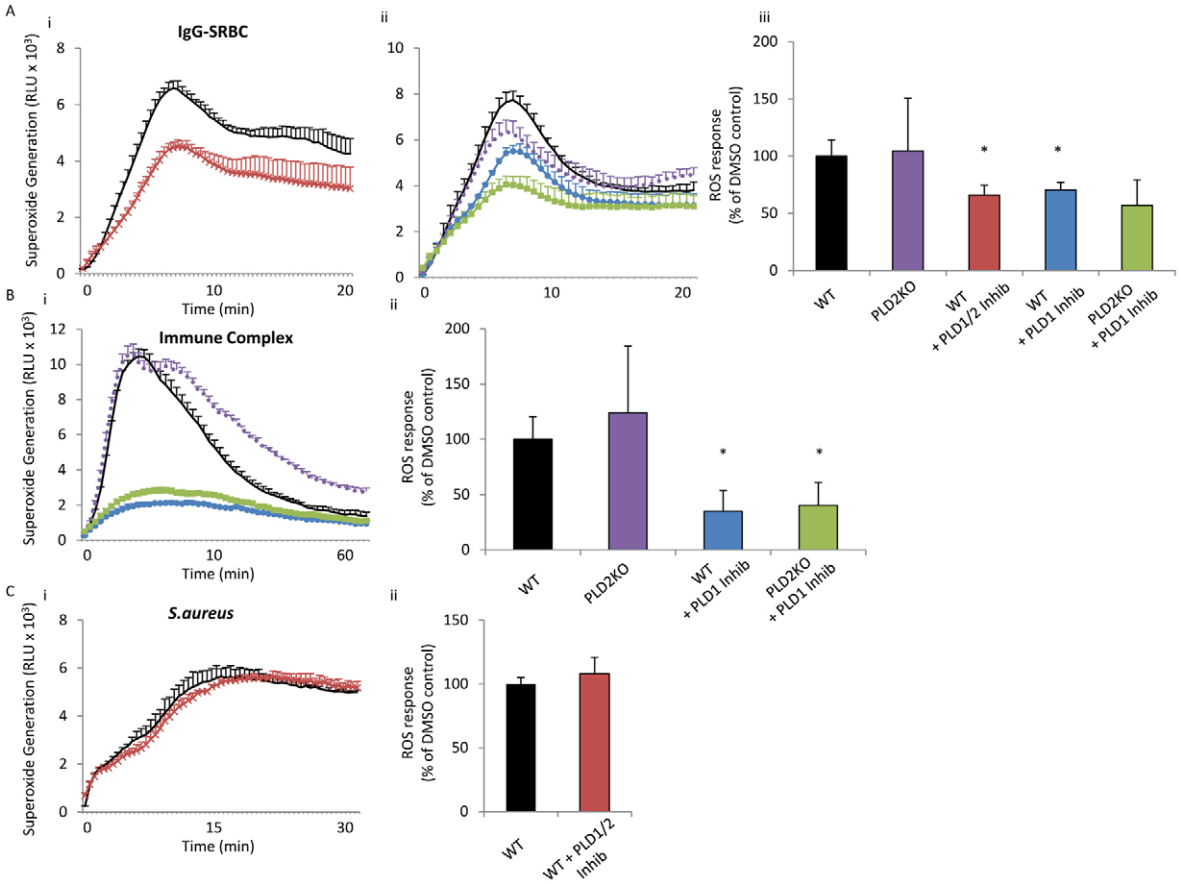

Fig. 4. PLD1 regulation of neutrophil ROS generation in response to Fcγ receptor stimulation not complement receptor stimulation. (**A**–**C**) Representative time course (i and ii in A, i in B and C) and total ROS generated (iii in A, and ii in B and C) from WT (black in i, ii, iii) and Pld2KO (purple in Aii and Aiii, and Bi and Bii) neutrophils incubated with or without PLD1/2 dual inhibitor (500 nM) (WT red in Ai and Aiii, and Ci and Cii) or PLD1 inhibitor (1 µM) (WT blue in Aii and Aiii, and Bi and Bii) upon stimulation with IgG-SRBC (20 minutes) (A), Immune complex (1:10,000 mouse anti-BSA antibody; 1 hour) (B) or *S. aureus* (1:20; 30 minutes) (C). Results are expressed as a percentage of individual WT controls and were collated from at least three independent experiments. IgG-SRBC: Pld2KO, $P = 0.720$; WT + PLD1/2 Inhib, $^*P=0.002$; WT + PLD1 Inhib, $^*P=0.014$; Pld2KO + PLD1 Inhib, $P=0.098$. Immune complex: Pld2KO, $P=0.590$; WT + PLD1 Inhib, $^*P=0.032$; Pld2KO + PLD1 Inhib, $^*P=0.046$. No P values were determined for *S. aureus*, although by observing scatter of line graphs, there do not appear to be any significant differences.

able to migrate (73% of WT cells reached at least a 50 μm horizon, as did 63% of WT cells treated with the PLD1/2 dual inhibitor) (Fig. 5A). Chemotaxis-stimulated directional migration was assessed in the EZ-Taxiscan chamber (Ferguson et al., 2007; Nishio et al., 2007) and to an fMLP-filled micropipette (data not shown) in response to a range of fMLP concentrations. Neutrophils pre-incubated with the PLD1/2 dual inhibitor were capable of directional migration towards the source of chemoattractant on both uncoated and fibrinogen-coated glass (1 μM fMLP; 40% of WT and 31% of WT cells treated with the PLD1/2 dual inhibitor reached a 50 μm horizon on glass; and 26% of WT and 39% WT cells treated with the PLD1/2 dual inhibitor cells reached a 50 μm horizon on fibrinogen) (Fig. 5B). Directionality, as measured by migratory index (how straight a cell moves, MI=distance from origin/total distance travelled), was unaffected in the presence of the PLD1/2 dual inhibitor [1 μM fMLP; MI=0.68± 0.033 (mean±s.e.) WT and 0.72±0.025 WT cells treated with the PLD1/2 dual inhibitor on fibrinogen].

Neutrophil adhesion to uncoated or fibrinogen- and pRGD-coated glass was assessed, and no significant difference in the number of cells that adhered (Fig. 5C) or in the surface area they covered (Fig. 5D) was found between WT and Pld2KO neutrophils (Fig. 5C,Di), or in WT cells treated with the PLD1/2 dual inhibitor (Fig. 5C,Dii). In keeping with this migration and adhesion data, no significant differences between WT cells treated with vehicle control or PLD1/2 dual inhibitor were observed when human neutrophil migration and adhesion under shear stress with or without perfusion of fMLP was assessed (Ding Luo and Gerard Nash, pers. comm.).

Discussion

The recent generation of pharmacological inhibitors of PLD (Monovich et al., 2007; Scott et al., 2009) and our generation of a Pld2KO mouse enabled us to study the role of individual PLD isoforms in neutrophil function. Our data show that PLD1 is required for efficient ROS production in response to (1) the GPCR agonist fMLP and the PKC activator PMA; (2) adhesion to integrin ligands; and (3) stimulation of Fcγ immune receptors. By contrast, neutrophils from Pld2KO mice are capable of assembling a functional NADPH oxidase complex and producing ROS in these situations. These data indicate a significant role for PLD1 in the immune response and importantly, a clear and novel distinction in the roles of the PLD isoforms in neutrophils. Interestingly and in contrast to literature-promoted expectations, we have also shown that Pld2KO neutrophils are still capable of many of the other functions required for an efficient immune response, such as chemotaxis, chemokinesis and adhesion to integrin ligands.

The results we present are surprising in the context of an accumulation of data over previous years which has implicated PLD in many neutrophil (or neutrophil-like) responses, such as polarisation of the actin cytoskeleton (Zouwail et al., 2005; Nishikimi et al., 2009; Su et al., 2009), migration (Zouwail et al., 2005; Lehman et al., 2006; Powner et al., 2007; Su et al., 2009; Knapek et al., 2010), adhesion and spreading (Iyer et al., 2006; Powner et al., 2007; Chae et al., 2008; Su et al., 2009) and production of ROS (Cadwallader et al., 2004; Levy et al., 2005). However, much of this published work was carried out using siRNAs (Iyer et al., 2006; Lehman et al., 2006; Chae et al., 2008; Knapek et al., 2010) or primary alcohols as inhibitors of PLD-mediated PA production (Cadwallader et al., 2004; Zouwail et al., 2005; Iyer et al., 2006; Powner et al., 2007; Chae et al., 2008; Knapek et al., 2010).

Primary alcohols such as butan-1-ol have been used in the study of PLD function for many years; they act as preferential nucleophilic acceptors in a transphosphatidylation reaction in which phosphatidyl PLD intermediates normally use water in the

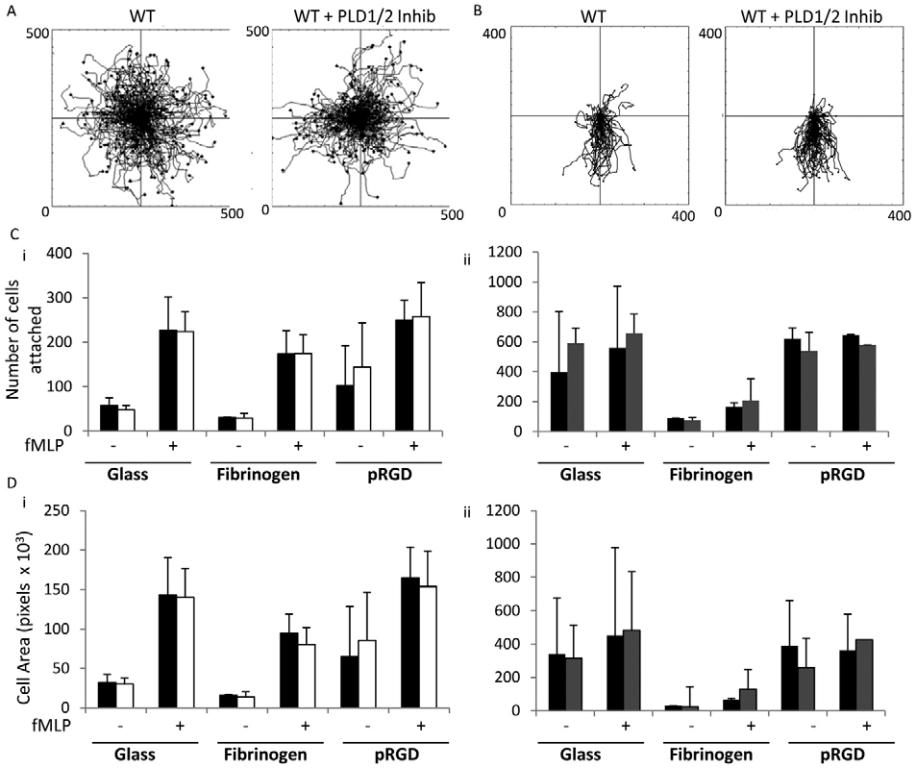

Fig. 5. Inhibition of PLD1 and PLD2 did not ablate neutrophil chemokinesis, chemotaxis or adhesion. (**A**,**B**) Centre zeroed tracks of individual WT neutrophils incubated with PLD1/2 dual inhibitor (500 nM) (right) or DMSO control (left) (A) stimulated with a bath application of fMLP (1 μM) on glass coverslips or (B) stimulated with an fMLP gradient in an EZTaxiscan chamber on fibrinogen-coated coverslips. The scale of each graph is in μm. Data are collated from (A) at least 320 cells from three movies from two individual independent experiments and (B) at least 215 cells from nine movies from four independent experiments. (**C**) Adhesion and (**D**) spreading of WT (black) incubated with PLD1/2 dual inhibitor (500 nM) (dark grey) (ii) or Pld2KO neutrophils (white) (i) to various surfaces stimulated with or without fMLP (1 μM). Results shown as (C) number of cells attached and (D) number of pixels total cell area covers. Results are from at least 24 fields of view from at least two independent experiments.

formation of PA, leading to the production of a phosphatidyl alcohol instead of PA. Thus the use of primary alcohols is an alternative substrate rather than an inhibition strategy. The phosphatidyl alcohol was thought to be inert, being unable to recruit or activate target proteins and downstream signalling. However more recent work has begun to raise concerns over potential off-target effects, or a lack of complete inhibition of PA production when using primary alcohols, such as butan-1-ol, to inhibit PLD (Skippen et al., 2002; Huang et al., 2005; Huang and Frohman, 2007; Su et al., 2009; Yanase et al., 2010). Consequently, previous work inferring that the presence of a primary alcohol led to the inhibition of PLD-dependent processes is now being brought into question, and in many cases, considerable re-evaluation is necessary. In addition to these drawbacks, PLD inhibition by primary alcohols does not present an opportunity to discriminate between any discrete roles of the two individual mammalian isoforms. More recently, PLD-isoform-selective siRNAs have been used to inhibit PLD activity. This is a more mid-term approach, with effects occurring in cultured cells over hours or days, and in many cases, the knockdown is incomplete. This could potentially increase the possibility of secondary effects on other signalling pathways; the use of short-acting PLD-isoform-selective inhibitors on freshly isolated primary cells or in long-term PLD-knockout mouse models removes this potential problem.

Our newly reported Pld2-knockout mouse model and a set of new generation isoenzyme-selective PLD inhibitors (Scott et al., 2009) provide an accurate tool to more reliably report on the roles of PLD in neutrophil function. We have clearly seen that the PLD inhibitors are on-target, reducing PA levels in freshly isolated neutrophils (Fig. 1B,C). Our data also imply that these inhibitors do not affect other major signalling pathways, such as the PI3K signalling pathway, because fMLP-induced ROS production is not inhibited in their presence. However, it should be noted that although the PLD inhibitors used in this study have been thoroughly tested and characterised with regard to isoform selectively (Scott et al., 2009), it is not impossible that these new generation inhibitors could themselves be shown to have non-specific effects in the future.

Despite the availability of PLD-selective inhibitors, our results do contradict some recent work from Frohman and co-workers (Su et al., 2009) and Nishikimi and colleagues (Nishikimi et al., 2009). Frohman and co-workers (Su et al., 2009) approached their study of PLD function by using a distinct small molecule PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) (Monovich et al., 2007). In agreement with our data, they also show that several biological processes blocked by butan-1-ol were not affected by FIPI (for example glucose-stimulated insulin release, regulatory exocytosis) [other examples have been reported (Yanase et al., 2010; Huang et al., 2005)]; however, they do still implicate PLD in neutrophil-like HL-60 transwell chemotaxis. It is important to note here that our study was carried out using freshly isolated bone-marrow-derived neutrophils and not a cell line, which could potentially explain some differences in results. Another possible reason for the discrepancies in these results could be explained by the use of a different inhibitor; Scott and colleagues (Scott et al., 2009) used a benzimidazole-containing scaffold as a starting point to generate a novel series of PLD1-selective inhibitors that are similar to the halopemide portion of the FIPI compound (Monovich et al., 2007). Other compounds used a different scaffold as a starting point for developing novel structure-activity relationships (Lavieri et al., 2009; Lavieri et al., 2010). Additionally, as described previously, there has been variation in reports of the involvement of signalling pathways in neutrophil chemotaxis that might originate from groups using different experimental conditions e.g. substratum, variations in cell priming and off-target effects of pharmacological inhibitors (Ferguson et al., 2007). Our work looking at the role of PLD2 uses the gold standard of a Pld2-knockout mouse model, and from this we can conclusively state that neutrophils lacking PLD2 are still capable of chemotaxis in the context that we tested. Nishikimi and colleagues (Nishikimi et al., 2009) implicate PLD-generated PA in stabilisation of the Rac guanine nucleotide exchange factor (GEF) DOCK2 at the leading edge of neutrophils; without this interaction, cells failed to form proper functional leading edges and perform chemotaxis inefficiently. Again, these results were obtained using the FIPI compound and studying PA binding to DOCK2 mutant proteins.

Despite the surprising lack of other PLD-dependent phenotypes in neutrophils, we clearly demonstrate a role for PLD1 in ROS production to (1) the soluble stimuli fMLP and PMA; (2) adhesion to integrin ligands; and (3) stimulation of Fcγ immune receptors.

Inhibition of both PLD isoforms in a quantitative chemiluminescence ROS assay led to an enhanced fMLP-induced and a diminished PMA-induced ROS response. Furthermore, this was shown in both cases to be due to inhibition of PLD1 not PLD2. The precise mechanism of PLD involvement in ROS signalling events downstream of both of these two stimuli is unclear. The differential effects of the GPCR agonist fMLP and the non-physiological PKC activator PMA are intriguing. PLD is not essential for ROS production downstream of the GPCR agonist fMLP, which is primarily regulated through PI3K activity, in fact, removing PLD activity appears to enhance ROS production in this setting. This could suggest a possible suppressive or regulatory role for PLD2 in signalling. Previous work has shown involvement of PLD in membrane trafficking (Bi et al., 1997) and receptor internalisation (Antonescu et al., 2010; Norambuena et al., 2010). Upon pharmacological inhibition of both PLD1 and PLD2, or inhibition of PLD1 in PLD2KO neutrophils, we have shown an enhanced fMLP-induced ROS response (Fig. 2). If PLD is required for fMLP receptor internalisation, inhibiting its activity could lead to the receptor being constitutively turned on at the membrane and an enhanced signalling response.

We observed that removal of PLD2 activity often led to an enhanced ROS response in many of the systems we assessed. An effective ROS response for bacterial killing and combating infection is important; however, uncontrolled overproduction of ROS by neutrophils can also be detrimental. Thus, confining the ROS response, both in space and time, is important to prevent self-damage. The failure to limit oxidase activation has a key role in pathological conditions such as acute respiratory distress syndrome (ARDS) and rheumatoid arthritis, while in the autoimmune disorder vasculitis, the neutrophil-induced tissue damage can lead to renal failure, lung damage and blindness, much of which is PA dependent (Williams et al., 2007). There is likely a role for PLD2 in ROS generation in other cell types, for example, many types of cancer cell, which have differing levels of PLD expression (Saito et al., 2007; Cho et al., 2008; Snider et al., 2010), also have increased levels of ROS (Szatrowski and Nathan, 1991; Toyokuni et al., 1995), which could lead to tumour heterogeneity, invasion and metastasis.

The cellular location of PLD is probably also important for its role in the ROS response. Brown and colleagues (Brown et al., 1998) previously showed that stimulation with PMA led to

translocation of PLD1 to the plasma membrane in RBL-2H3 cells. Our results show a requirement of PLD1 for PMA-induced oxidase formation. PLD1 could be necessary for the translocation of cytoplasmic components of the oxidase, such as $p47^{phox}$ (a putative PA binding site has been previously reported in the PX domain of $p47^{phox}$) (Karathanassis et al., 2002) to the membrane, or it might be required at the membrane for full functioning of the oxidase complex. We attempted to address the potential importance of PLD in the localisation of oxidase formation by carrying out assays to distinguish between the relative amounts of extracellular and intracellular ROS formation in the various stimulations we have used in the study (supplementary material Fig. S2B) and also a visual analysis of localisation of active oxidase complex using a Nitroblue tetrazolium (NBT)-formazan assay (supplementary material Fig. S2C). Neither of these assays provided us with a PLD-dependent requirement for either intracellular or extracellular ROS formation exclusively (under the conditions we used, most of the ROS produced were extracellular) or a specific location of the ROS formation within the cell.

Our results imply connections in the signalling pathways required for functional ROS responses, in particular the links between PLD and PKCs (Kim et al., 1999b; Kim et al., 1999a; Ghelli et al., 2002; Hu and Exton, 2003; Oka et al., 2003; Hu and Exton, 2005) and between PA and the oxidase component $p47^{phox}$ (Karathanassis et al., 2002). PKCs are important for the phosphorylation of the oxidase components $p40^{phox}$ and $p47^{phox}$ (Someya et al., 1999; Dekker et al., 2000; Dang et al., 2001; Fontayne et al., 2002; Bey et al., 2004; Lopes et al., 2004; Yamamori et al., 2004; Cheng et al., 2007). There is a lack of clarity in the literature as to which PKC isoforms are important for phosphorylation of oxidase components downstream of fMLP and PMA stimulation. Cheng and colleagues (Cheng et al., 2007) describe a crucial role for PKCδ in fMLP-induced phosphorylation of $p47^{phox}$ and activation of the oxidase, whereas Dekker and co-workers (Dekker et al., 2000) implicate PKCβ with a lesser role for PKCδ in PMA and FcγR-induced ROS production. Phosphorylation of $p40^{phox}$ (T154) has been shown to be entirely PKCδ dependent downstream of PMA, but a combination of cPKCs (α and/or β) and PKCδ are required downstream of fMLP (Chessa et al., 2010). Regardless of the particular PKC isoform involved, one could foresee that if PLD1 lies downstream of, or was required for the PKC-dependent phosphorylation of $p47^{phox}$ (Karathanassis et al., 2002) then PMA stimulation when PLD1 was inhibited would lead to diminished oxidase activation and ROS production (as seen in our data). By contrast, when the oxidase is activated through an additional pathway, such as when PI3Kγ signalling is activated by fMLP receptor occupation, blocking PLD1 activation does not inhibit ROS generation. The small enhancement observed when PLD is inhibited might be due to effects upon the balance between fMLP, PKC and $p47^{phox}$, or $p40^{phox}$ phosphorylation. Under these conditions, inhibiting PLD activity and its binding to or effect on PKC might lead to dysregulation and enhancement of ROS production.

Inhibition of PLD1 led to decreased ROS production upon adhesion to a fibrinogen and pRGD-coated surface. We also found that PLD1 inhibition led to a defect in ROS production upon immune complexes and to IgG–SRBC, both of which signal through Fcγ receptors (FcγRIII and FcγRIV) (Jakus et al., 2008; Nimmerjahn and Ravetch, 2008). This suggests that PLD1 is involved in signalling downstream of Fcγ receptors. Integrins and Fc receptors signal through a similar immuno-tyrosine activation motif (ITAM)-based receptor proximal tyrosine phosphorylation cascade (Jakus et al., 2007). Src family tyrosine kinases phosphorylate tyrosine residues within ITAMs of receptors. These phosphorylated sites then become docking sites for Src homology 2 (SH2) domain-containing proteins, such as the Syk family and PI3K, which then activate numerous effectors in the cell (Sanchez-Mejorada and Rosales, 1998; Ravetch and Bolland, 2001; Joshi et al., 2006). Signalling downstream of ITAM tyrosine phosphorylation activates both the PLCγ–DAG–PKC pathway and the PI3K pathway. The generated DAG will be able to activate PKC, whereas the PI3K lipid product PIP3 can activate the exchange factors ARNO, Tiam1 and Vav, which will in turn promote GTP loading and thus activation of Arf6 and Rac. We have previously demonstrated that ligation of the IgE receptor, which promotes tyrosine kinase activity in RBL-2H3 cells, stimulates PLD1 activity in a PI3K-dependent manner cooperatively through PKC, Rac and Arf6 (Powner et al., 2002). Neutrophil activation by both fMLP and Fcγ receptors involves stimulation of PI3K signalling, however, the GPCR is coupled to the γ-isoform whereas Fcγ activates both the β- and δ-isoforms. This might suggest that PLD1 activation is downstream of the PI3Kβ and PI3Kδ isoforms, but not the PI3Kγ isoform; alternatively, the localisation of these isoforms might affect the oxidase differently, with translocation of the activated complex, which could be PLD1 dependent, becoming more crucial following Fcγ stimulation.

Thus we conclude that PLD1, not PLD2, regulates phorbol-ester-, chemoattractant, adhesion-dependent- and Fcγ-receptor-stimulated ROS production, whereas other neutrophil functions, such as chemotaxis and adhesion can occur independently of PLD (Fig. 6). We surmise that the PLD field has been confused by the use of primary alcohols as inhibitors of PLD activity and now that gold standard PLD mouse models are available, some of the work on PLD function might need to be reassessed.

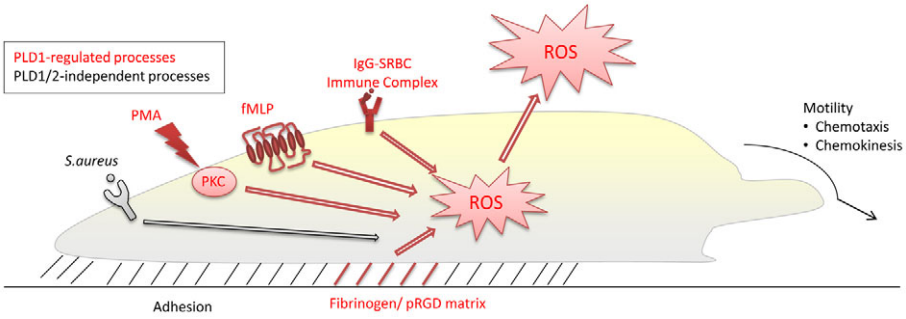

Fig. 6. Summary of PLD1 regulation of neutrophil processes. An illustration of a neutrophil showing that PLD1, not PLD2, regulates adhesion-dependent ROS production stimulated by phorbol ester, chemoattractant and Fcγ receptor (shown in red), whereas other neutrophil functions such as chemotaxis, adhesion and ROS production in response to serum opsonised *S. aureus* can occur independently of PLD (shown in black).

Materials and Methods

Materials

All materials used were of the lowest endotoxin level available and were purchased from Sigma unless stated otherwise. PLD inhibitors have been previously described (Scott et al., 2009). Mouse strains: The Pld2KO was generated using ES cells which carry a *Pld2* null allele through deletion of exons 13–15 on chromosome 11. The excision comprises part of the PH domain and the catalytic HxKxxxxD motif. *Pld2* mutant cells were expanded and injected into mouse blastocyts and following chimera generation, subsequent matings showed germline transmission and southern blotting demonstrated that PLD2-deficient mice were viable and did not express any *Pld2* mRNA. Animals were rederived, established and housed under barrier control at Babraham Institute Small Animal Barrier Unit.

Isolation of mouse neutrophils

Murine bone marrow was dispersed in HBSS (without Ca^{2+} and Mg^{2+}) with 0.25% fatty acid-free bovine serum albumin (BSA) (HBSS/BSA) and was centrifuged (1256 ***g***, 30 minutes, room temperature) over discontinuous 62–55% Percoll gradient in HBSS. Mature neutrophils were obtained from the interface (purity, 75–85% by cytospin), and contaminating red blood cells were removed by Geyes lysis (130.8 mM NH_4Cl, 5.0 mM KCl, 0.78 mM Na_2HPO_4, 0.17 mM KH_2PO_4, 5.56 mM glucose, 1.04 mM $MgCl_2.6H_2O$, 0.28 mM $MgSO_4.7H_2O$, 1.53 mM $CaCl_2.2H_2O$ and 13.39 mM $NaHCO_3$); cells were washed three times in HBSS/BSA. Neutrophils were resuspended in D-PBS with Ca^{2+} and Mg^{2+} (D-PBS+) supplemented with 1 g/l glucose, 4 mM sodium bicarbonate (D-PBS++) for assays.

Western blotting

Whole WT and PLD2KO brains were collected and immediately frozen in liquid nitrogen. Brains were stored at –80°C until they were fractured while frozen using a BioPulveriser (BIOSPEC) that had been thoroughly pre-chilled in liquid nitrogen. The brain powder was transferred into lysis buffer (100 mM Tris-HCl, 9 M urea, pH 7.5) and incubated at 55°C for 30 minutes. Insoluble material was removed by centrifugation (16,000 ***g***, 30 minutes), and the soluble material transferred to a fresh tube. The final urea concentration was adjusted to 3 M. The protein content was determined using a BCA protein assay kit (Pierce) and equal amounts of brain material were analysed by standard 6% SDS-PAGE and western blotting techniques. PLD2 was detected using a rat polyclonal antibody generated against the C-terminus of mouse PLD2 (aa 613–933). Neutrophil pellets from WT and Pld2KO mice were analysed by standard SDS-PAGE and western blotting techniques. PLD1 was detected using a PLD1 antibody (#3832, Cell Signalling). Expression of NADPH oxidase cytoplasmic components were detected using antibodies against $p40^{phox}$ (#07-501, Upstate), $p47^{phox}$ (#07-500, Upstate) and $p67^{phox}$ (#07-502, Upstate).

Adhesion protein or immune complex immobilisation

Fibrinogen- (150 μg/ml) and poly-RGD-coated (20 μg/ml) surfaces were adsorbed onto wells of 96-well polystyrene luminometer plate (no:23300 Berthold Technologies Ltd) or glass coverslips overnight at 4°C (fibrinogen) or at room temperature for more than 2 hours (pRGD). Immune complexes were immobilised by coating plates overnight with PBS containing BSA (100 μg/ml). Wells were blocked with 1% fat-free milk for 45 minutes at room temperature. Immune complexes were formed by addition of 1:10,000 dilution of mouse anti-BSA antibody for 1 hour at room temperature and excess was washed three times with PBS.

Opsonisation of particles

S. aureus Wood 46 bacteria were grown, opsonised with DPBS+ and 10% mouse serum (isolated as described previously (Ellson et al., 2006), and prepared as described previously (Anderson et al., 2008). 10 μl of sheep red blood cells (SRBCs) in Alsevers (TCS Biosciences) were washed (1500 ***g***, 4 minutes) and resuspended in 1 ml D-PBS with 0.1% fatty-acid-free BSA. SRBCs were opsonised with 1/1000 dilution of rabbit anti-SRBC IgG (MP Biomedicals), rotating end-over-end at room temperature for 20 minutes. SRBCs were washed twice and resuspended in 800 μl of D-PBS++.

Detection of ROS

Neutrophils were resuspended at 6.25×10^6/ml. Cells were primed with TNFα (1000 U/ml) and GMCSF (100 ng/ml; R & D systems) for 1 hour and pre-incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%) in the presence of luminol (150 μM final concentration) and HRP (18.75 U/ml final concentration) for final 10 minutes at 37°C before stimulation. Neutrophils (5×10^5/well) were added manually to a luminometer plate pre-coated with adhesion proteins (with or without addition of TNFα (20 ng/ml final concentration); immune complex; containing fMLP (1 μM final concentration); PMA (100 nM); *S. aureus* (1:20 ratio); or IgG-SRBC, and measurement of light emission was started immediately and recorded by a Berthold MicroLumat Plus luminometer (Berthold Technologies) maintained at 37°C. Data were output as relative light units per second. To detect intracellular versus extracellular ROS, cells were incubated with either luminol (150 μM) and SOD (375 U/ml) (intracellular); isoluminol (150 μM) and HRP (18.75 U/ml) (extracellular) or luminol/HRP (total) for 10 minutes at 37°C before stimulation.

PA measurements

Neutrophils were resuspended at 2×10^7/ml. Cells were primed with TNFα and GMCSF for 1 hour and pre-incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%). Cells were stimulated with PMA (100 nM, 10 minutes) at 37°C. Stimulation was quenched by addition of excess volume of ice-cold D-PBS++. Cells were pelleted at 1500 ***g*** for 2 minutes, aspirated dry, resuspended in ice-cold methanol, 0.88% NaCl and chloroform (in a 1: 1:2 ratio) and internal standards were added (200 ng 12:0 PA). Phases were thoroughly mixed, incubated on ice for 5 minutes and centrifuged at 300 ***g*** for 5 minutes to split the phases. Lower phase was collected and upper phase re-extracted. Lipid extracts were dried on a vacuum centrifugal evaporator at room temperature, resuspended in a 1:1 solution of chloroform and methanol, and 17 μl was injected for analysis by LCMS [Shimadzu IT-TOF LC/MS/MS system hyphenated with a five-channel online degasser, four-pump, column oven, and autosampler with cooler Prominence HPLC (Shimadzu)]. Accurate mass (with mass accuracy 5–10 p.p.m.) and tandem MS were used for molecular species identification and quantification. The identity of lipid was further confirmed by reference to appropriate lipids standards. All the solvents used for lipids extraction and LC/MS/MS analysis were LC-MS grade from FisherScientific.

Nitroblue tetrazolium (NBT) formazan assays

Neutrophils were resuspended and primed as for ROS assay and pre-incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%) and 0.2 mg/ml NBT, for final 10 minutes at 37°C. Cells were stimulated with fMLP (1 μM final; 3 minutes); PMA (100 nM; 10 minutes) or IgG-SRBC (10 minutes) at 37°C. Non-phagocytosed SRBC were lysed by Geyes addition for 1 minute on ice. Cells were allowed to adhere onto glass coverslips, fixed with 4% paraformaldehyde and mounted. Dark formazan deposition was detected by DIC imaging on a Zeiss LSM 510 META point-scanning confocal microscope with an integrated camera, using a Plan/Apochromat 63×/1.4 oil objective.

Neutrophil chemokinesis

Neutrophils were resuspended at 5×10^5/ml. Cells were allowed to adhere to glass coverslips with or without adhesion proteins (see above) for 10 minutes and then incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%) for a further 10 minutes at 37°C. Cells were stimulated with a bath application of fMLP (1 μM), cell migration was recorded every 30 seconds for 30 minutes using an Axiovert 200 microscope with a 5× objective (Zeiss, Welwyn Garden City, UK).

Neutrophil chemotaxis

Neutrophils were resuspended at 3×10^5/ml and incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%) for 10 minutes at 37°C. The EZ-Taxiscan chamber (Effector Cell Institute, Tokyo, Japan) was assembled as previously described (Nishio et al., 2007). Cell migration was recorded every 30 seconds for 30 minutes using a BD Pathway 855 system (BD Biosciences) (with an individually manufactured adapted stage insert) and was maintained at 37°C in an environmental chamber.

Neutrophil adhesion

Neutrophils were resuspended at 2×10^6/ml and pre-incubated with or without PLD inhibitors or vehicle control (DMSO, 0.1%) for 10 minutes at 37°C. Cells ± fMLP (1 μM final concentration) were allowed to adhere to glass coverslips coated with or without adhesion proteins (see above) for 15 minutes at 37°C and fixed with 4% paraformaldehyde, stained with antibody against FITC-Gr-1 (RB6-8CS BD Pharmingen) and mounted. At least 12 fields of view were recorded, number of cells attached and cell area were detected and recorded using Volocity software (Perkin Elmer).

Analysis of migration movies

The *x,y* coordinates of each cell were measured using Metamorph (Molecular Devices, Sunnyvale, UK) and were corrected using Mathematica (Wolfram Research, Long Hanborough, UK) (Zicha et al., 1997). The population of motile cells was then used to calculate the percentage of motile cells, speed and the migratory index (distance from origin/total distance travelled).

Statistical analysis of ROS data

The ratio of Pld2KO (or other condition) over WT was calculated. It was assumed that samples came from a normally distributed population and that the variability between the groups was similar by looking at the scatter of the data and so parametric one sample *t*-tests were applied. Data significantly different from WT ($P<0.05$) is shown by asterisk and *P* values are quoted in figure legends.

Statisitical analysis of PA measurements

The ratio of *Pld2* null over WT was calculated, values per paired comparison were compared to 1 and one-sample *t*-tests were applied. Comparisons were not significant and *P* values are quoted in figure legends. Owing to relatively small sample size and for completeness, an equivalent non-parametric one-sample *t*-test (one-sample Wilcoxon signed rank test) was carried out. This gave the same results: comparisons were not significant.

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Supplementary material available online at
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