Phospholipid signaling in innate immune cells.

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Abstract

Phospholipids comprise a large body of lipids that define cells and organelles by forming membrane structures. Importantly, their complex metabolism represents a highly controlled cellular signaling network that is essential for mounting an effective innate immune response. Phospholipids in innate cells are subject to dynamic regulation by enzymes, whose activities are highly responsive to activation status. Along with their metabolic products, they regulate multiple aspects of innate immune cell biology, including shape change, aggregation, blood clotting, and degranulation. Phospholipid hydrolysis provides substrates for cell-cell communication, enables regulation of hemostasis, immunity, thrombosis, and vascular inflammation, and is centrally important in cardiovascular disease and associated co-morbidities. Phospholipids themselves are also recognized by innate-like T cells, which are considered essential for recognition of infection or cancer, as well as self-antigens. This review will describe the major phospholipid metabolic pathways present in innate immune cells and summarize the formation and metabolism of phospholipids as well as their emerging roles in cell biology and disease.

Main Text

Circulating innate immune cells, including neutrophils, platelets, monocytes, eosinophils, and mast cells, are considered resting basally. However, they rapidly activate during injury, such as bleeding or trauma, to trigger hemostasis and prevent bacterial invasion. Major metabolic changes to the lipid pool that accompany innate immune activation are essential in orchestrating an effective innate immune response and then initiating the process of wound healing and repair. This cellular activation is exemplified by multifaceted alterations to the phospholipid (PL) pool and includes: (i) membrane remodeling and generation of potent bioactive signaling mediators from PL-derived substrates (lysophospholipids (lysoPL), oxylipins, and platelet-activating factor (PAF) etc.); (ii) aminophospholipid (aPL) externalization, providing a negatively-charged membrane that supports clotting factor activities on the cell surface; (iii) generation of large numbers of enzymatically-oxidized PL (eoxPL), which are required for hemostasis, immediately upon innate immune cell activation; and (iv) inside the cell, PL headgroup phosphorylation that occurs within seconds, leading to formation of transient membrane anchors for kinases that regulate GPCR signaling, endocytosis, apoptosis, and cytokinesis.

Separate from changes to the PL pool, lipid-reactive T cells restricted to the CD1 family of antigen-presenting molecules, can sense the presence of bacterially-derived lipids, self-lipids during thymic selection, and self-lipids that may be perturbed during cellular stress [1, 2]. Many lipid-reactive T cells appear non-reactive to common phospholipids normally found within the cell membrane. However, T cells reactive to bacterially-derived phospholipids [3, 4], rare phospholipids [5], and lysophospholipids expressed on certain tumors [6, 7] have been described. These processes will be described in detail below.

PLs represent a diverse group of lipids also known as glycerophospholipids (GP, described using LIPID MAPS nomenclature). The five classes are subdivided based on polar headgroup and include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS). Within each class, molecular species are differentiated by fatty acid (FA) substitution at the *sn1* and *sn2* positions of the glycerol backbone (Figure 1A). Generally, *sn1* FAs are saturated or mono-unsaturated, whereas *sn2* are polyunsaturated (PUFA) with longer acyl chains. While the *sn2* FA is always an acyl substituent, the *sn1* for PE or PC can be either an alkyl-linked (ether-linked) or 1Z-alkenyl-linked (plasmalogen), with the latter particularly abundant in circulating immune cells (Figure 1B). Thus, blood cells contain literally hundreds of unique PL species, although the most quantitatively abundant contain a relatively restricted set of FAs: palmitic acid (PA, 16:0), stearic acid (SA, 18:0) or oleic acid (OA, 18:1) at *sn1*, and linoleic acid (LA, 18:2), arachidonic acid (AA, 20:4), eicosapentaneoic acid (20:5, EPA) or docosahexanoic acid (22:6, DHA) at *sn2* (Figure 1 C,D). Overall, AA is present in PLs at least at 10-fold higher concentrations than other PUFAs at *sn2* in immune cells. Dietary intake of fatty acids, through ingesting high doses of fish oils, can influence relative levels of omega 3 (DHA, EPA) versus omega 6 (AA, LA) fatty acids in blood cell PLs[8, 9]. Whether this leads to changes in PL-mediated signaling in the innate immune system is unknown. However it has knock-on effects on levels of oxylipins generated in serum, with higher levels of some omega3 derived species observed [10-15].

Phospholipids are synthesized and remodeled through several interconnected enzyme pathways, including the Kennedy pathway (PC, PE synthesis), the cytidine diphosphate diacylglycerol (CDP-DAG) pathway (synthesis of PS and PI from PA), and the Lands cycle (FA removal and reattachment to PLs) (Supplementary Figure 1 A,B) [16-18]. These are common to all cell types, including circulating blood cells, and are subject to dynamic regulation depending on metabolic state. The Lands cycle controls substrate supply for bioactive lipid signaling and is described in more detail below.

Following an acute injury, circulating cells become trapped at the wound site. The resulting clot composition is highly dependent on vessel type and co-existing inflammation [19-21]. Concurrently, trapped cells are exposed to soluble protein and non-protein mediators that activate receptor-dependent processes, including tissue factor, collagen, thrombin, ADP, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs). PAMPs include bacterial products such as lipopolysaccharide (LPS) and f-met-leu-phe (fMLP), both of which stimulate cells in the nanogram range. LPS acts via TLR complexes, while fMLP mediates signaling via a GPCR called FPR1. At the same time, a cascade of cell activation processes characterized by extracellular calcium influx and release from intracellular stores are associated with PL hydrolysis. This is mediated by several types of phospholipases, broadly categorized as PLA_1, PLA_2, PLC, PLD and named by site of PL hydrolysis. Each comprises families of multiple isoforms (Figure 2 A). Many different phospholipases are acutely switched on in activated blood cells, and their coordinated activities are responsible for initiating cell activation during the injury response. This controls much of the signaling that leads to lipid membrane remodeling and a plethora of lipid-dependent bioactivities, both intracellular and extracellular. Lipids generated during initial phospholipase activity are either released as secondary paracrine mediators (e.g. oxylipins), or retained in the cells to act locally, either physically changing membrane structure (e.g. eoxPL) or signaling at intracellular nuclear receptors (e.g. FA activation of PPARs). The following sections describe PL metabolism in innate immune cells in more detail.

1. PLA_2-mediated phospholipid hydrolysis forms lysoPL and FAs.

Pathophysiological activation of innate cells triggers immediate cell membrane hydrolysis by phospholipases. The best studied are PLA_2 isoforms, which comprise ~16 groups and several subgroups, with six main PLA_2 subtypes [22]. PLA_2 removes fatty acyl groups from the *sn2* position of PL, leading to lysoPL and FA generation and thereby supporting the first step in bioactive oxylipin formation (Figure 2 A). Several lysoPLs are generated during platelet activation, including lyso-PE, -PC, -PI and –PS [23]. Leukocytes and platelets express at least three classes of PLA_2: secretory ($sPLA_2$, Groups II and X), calcium-dependent ($cPLA_2$, Group IV), and calcium-independent ($iPLA_2$, Group VI), whilst several others are known to exist in other cells. Another PLA_2 isoform, termed PAF-acetyl hydrolase (PAF-AH), is found in the circulation [24].

Regulation of innate immune cell PLA_2 is multifaceted and complex. For example, in monocytes, both MAPK-catalyzed phosphorylation and PKC activate $cPLA_{2}\alpha$ [25]. For many years, $cPLA_{2}\alpha$ was considered the primary source of AA for eicosanoid generation, but recent studies show a major role for $iPLA_2$ in several circulating cell types. In this regard, platelet $cPLA_{2}\alpha$ can catalyze AA release from innate immune PL pools in response to collagen, enabling generation of thromboxane A_2 and 12-HETE [26]. However, $iPLA_{2}\gamma$ is required to mobilize AA from plasmalogen PEs and PGs for ADP-induced thromboxane B_2 generation [27]. Thus, different PLA_2 isoforms couple to distinct receptor pathways, though both can facilitate platelet eicosanoid generation. Similarly, in leukocytes, cPLA2α and iPLA2β act together to provide different signaling lipids at separate intracellular sites, with both required for migration in response to MCP-1 (also known as CCL2) [28].

Importantly, PLA_2 provide the essential link between PL metabolism and cyclooxygenase-mediated (COX-mediated) pathways in immune cells, e.g. generation of thromboxane in platelets, and prostaglandin E_2 (PGE_2) in inflammation, both of which require AA. COX-1 and COX-2 are the well-known targets of aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), and more recently-developed COX-2-selective NSAIDs, underscoring their clinical importance in vascular and chronic inflammatory disease.

Circulating blood cells express several $sPLA_2$ isoforms, including Group IIA (platelets, leukocytes), Group X (neutrophils), and Group III (mast cells). Group IIA $sPLA_2$ attacks bacterial membranes during host defense in innate immunity, while Group III targets stromal fibroblasts. PG and PE are the primary targets of Group IIA, with host cells being relatively protected since PC comprises most of the external membrane leaflet of healthy cells. In contrast, in mast cells, Group III $sPLA_2$ attacks stromal cells via AA release and its metabolism to prostaglandin D_2, promoting mast cell maturation and ultimately anaphylaxis [29].

Hydrolysis of the *sn1* fatty acyl group can also result in lysoPL and FA release, in this case due to PLA_1 (Figure 2 A). Little is known about PLA_1 in immune cells, although a fast release of saturated FAs occurs upon platelet activation [30]. A platelet PLA_1 isoform was purified in 2011 and proposed as a source of circulating lysoPA generated during clotting, acting in concert with autotaxin to mediate sequential hydrolysis of cellular PC [31].

Regulation of PLA_2 isoforms in innate immune cells is complex, given the large number of gene products, varied cell expression, intracellular localization, activation, control, etc. For further information on this topic, the reader is directed towards several recent reviews [25, 29, 32].

Phospholipases and their products play multiple and complex roles in disease, with symptoms noted well beyond the innate immune system. Deficiency of cPLA$_2\alpha$ in humans leads to major gastrointestinal complications including bleeding, as well as increased susceptibility to infection, and acute respiratory distress syndrome. A lack of platelet thromboxane B2 is associated with platelet defects[25]. On the other hand, mice lacking cPLA$_2\alpha$ are protected from hepatic liver deposition and fibrosis[25]. Deficiency of iPLA$_2\beta$ is linked with several diseases including neurological, cancer, vascular and immune[32]. In terms of inflammation, iPLA$_2\beta$ is required for macrophage spreading/adhesion[32]. Inhibitor studies have shown that cPLA$_2$ and iPLA$_2$ isoforms are critical for development of autoimmune disease, such as experimental autoimmune encephalomyelitis[32].

2. Scramblase activities leading to aPL externalization.

Normally, circulating blood cells maintain an asymmetric plasma membrane with PC the most abundant externally-facing PL and PE/PS primarily located on the cytosolic surface. PE and PC each make up about 40% of total PL in blood cells, with PS being only a few mol % [33, 34]. Maintaining asymmetry is an energy-requiring process utilizing flippase and floppase enzymes to prevent exposure of the negatively-charged aminophospholids (aPL) PS and PE on the outside of the cells. Upon activation and calcium mobilization, platelets rapidly activate scramblase, leading to externalization of aPL (Figure 2B) [35, 36]. This changes the biophysical nature of the membrane to allow coagulation factors to bind and work effectively to generate fibrin. A genetic defect in aPL externalization has been described, Scott Syndrome, that manifests as a relatively mild bleeding phenotype[37]. This has been localized to mutations in TMEM16F, a protein proposed to play dual roles as both a channel and scramblase[38]

Coagulation factors comprise a series of serine proteases and associated co-factors that are largely inactive in the fluid phase of blood. In order to facilitate clot formation, they must self-associate on a negatively-charged surface, where they reach a high local concentration. This is classically achieved by platelets externalizing PE/PS following agonist activation [35, 36]. The headgroup of PS facilitates calcium binding to the surface, which ultimately allows binding of negatively-charged gamma-carboxyglutamic (Gla) domains of factors at the cell membrane (Figure 2B). The ability of PS to support Gla-domain-binding is enhanced by PE and eoxPL, a recent discovery that is described in more detail later [39, 40]. Platelets contain several molecular species of PE and PS. The optimum FA composition to support coagulation is around 18-20 carbons, with typical unsaturation seen in platelet PLs, though plasmalogen and acyl forms of PE are equally effective in this context [41]. Recent studies show that PE and PS associate on the surface of platelets in defined microdomains, forming “cap” structures [42]. This may further enhance hemostasis by reducing the surface area available, leading to higher local concentrations of coagulation factors on the cells.

PE/PS externalization is often measured using annexin V, which binds to negatively-charged membranes via electrostatic interactions, similarly to coagulation factors [43-45]. Often, annexin V positivity is described as PS externalization, but it can also bind PE. Another protein, lacthadherin, is reportedly PS-specific [46, 47]. During inflammation, apoptotic innate immune cells can lose membrane asymmetry, also leading to PE/PS externalization [34]. This is particularly relevant in the case of neutrophils, which undergo apoptosis following infection. Neutrophil apoptosis occurs alongside elevated coagulation during systemic infection and sepsis, suggesting a mechanistic link between neutrophil aPL exposure and associated coagulation [48].

3. Generation and action of eoxPL in innate immunity.

Oxidized PCs with truncated FAs containing reactive aldehydes at the *sn2* position have long been known to be generated non-enzymatically [49-52]. They are detected in atheroma lesions and in the circulation during a myocardial infarction caused by plaque rupture [53]. In contrast, several families of enzymatically-oxidized PL (eoxPL), comprising eicosanoids/prostaglandins attached to PLs, were recently discovered. Circulating blood cells (neutrophils, platelets, eosinophils) and resident murine peritoneal macrophages generate eoxPL in a controlled manner by as part of the innate immune response [40, 54-59]. They form via the catalytic activities of lipoxygenases (LOX) as well as COX-1 (Supplementary Figure 2A). The most abundant eoxPL are PEs, but PC forms are also found, and low levels of PI analogs were recently detected in platelets [60]. PC and PE eoxPL form as both plasmalogen and acyl species.

For many years, LOX isoforms were best known to generate monohydroperoxide derivatives of free-acid PUFAs, primarily AA (HpETEs), but also DHA (HpDOHEs) and LA (HpODEs). Cellular glutathione peroxidases (GPx) rapidly reduce lipid hydroperoxides to form hydroxyeicosatetraenoic acids (HETEs), hydroxydocosahexanoic acids (HDOHEs), and hydroxyoctadecadienoic acids (HODEs), respectively. Positional isomers of these are generated in a cell-specific manner due to LOX expression patterns, e.g. by 5-LOX (5-HETE in neutrophils, monocytes), 15-LOX (15-HETE, 17-HDOHE in human eosinophils), and 12-LOX (12-HETE, 14-HDOHE in platelets). In mice, 15-LOX is represented by a 12/15-LOX gene product, with a similar expression pattern to human cells, that generates primarily 12-HETE and 14-HDOHE. This isoform is also highly expressed in murine peritoneal macrophages and is inducible by Th2 cytokines. Since AA is present in PL in at least 10-fold higher concentrations in immune cells than other PUFAs, its monohydroxylated products are the most abundant; for example, 12-HETE is the predominant eicosanoid generated by human platelets.

Most LOXs will only utilize free-acid PUFA due to steric hindrance at the active site; however, the 15-LOX (12/15-LOX in mice) can uniquely utilize oxidize PLs [61]. Thus, eoxPLs generated via 5- or 12-LOXs require a cycle of hydrolysis and re-esterification, involving PLA_2 and MBOAT/LPAT activities, while those from the 15- or 12/15-LOX do not (Supplementary Figure 2B) [40, 56, 58]. This is consistent with observations that neutrophil or platelet HETE-PLs are generated following agonist activation of cells to trigger PLA_2, while eosinophil/macrophage HETE-PLs are detected under basal conditions [40, 56, 58, 59]. Similarly, eoxPL containing COX-1 products, such as prostaglandin E_2–PEs, require a cycle of hydrolysis and re-esterification [54].

Circulating innate immune cells and platelets generate eoxPL via coordinated signaling pathways mediated by receptor-dependent calcium mobilization within 2-5 minutes of activation [40, 54, 56]. Unlike free-acid oxylipins, the cells retain eoxPL within the membrane compartment and do not secrete them, suggesting a different biological role to their free analogs. Up to 2016, only small numbers of eoxPL were known, typically 4-6 per cell type. However, studies using high-resolution lipidomics recently showed that human platelets generate over 100 unique molecular forms [30]. The most abundant are 12-HETE-PLs, in line with the relative abundance of platelet 12-HETE. However, multiple oxidized forms of AA are detected in platelet eoxPL, along with mono-hydroxylated forms of rarer FAs (DHA, EPA, and others) [30]. EoxPLs that contain thromboxane B2 have never been detected, despite the abundance of this platelet lipid, suggesting that not all oxylipins are substrates for re-esterification. A recent study demonstrated that specific eicosanoids serve as substrates for acyl-CoA synthetases, which activate eicosanoids to produce an eicosanoid-CoA species. This eicosanoid-CoA species then can be utilized by an acyltransferase (like MBOATs) to esterify the eicosanoid into PL species, indicating that cellular control of eoxPL formation may occur at the level of esterification [62]. Furthermore, correlation analysis of eoxPL from platelets shows clustering into structurally-related groups, further supporting the idea that their formation is dictated at least in part by the oxidized FA structure and resulting specificity of esterification pathways [39].

eoxPLs display multiple biological activities relevant to innate immunity and inflammation regulation. In neutrophils, HETE-PEs promote antibacterial activities, including enhancing IL-8 release and NET formation, while macrophage KETE-PEs are low affinity agonists for PPARγ [56, 57]. Recently, HETE-PEs and -PCs were shown to be potently pro-coagulant due to their enhancement of PS binding to coagulation factors (Supplementary Figure 3A) [39]. In this, the lipids' oxidation epitope enhances the overall electronegative character of the plasma membrane, facilitating calcium interactions with PS that are required for blood clotting. Related to this, eoxPL are significantly elevated in circulating cells in patients with the thrombotic disorder antiphospholipid syndrome (aPLS), and mice lacking either platelet or eosinophil LOX isoforms display a venous bleeding defect that can be corrected by eoxPL administration [39, 63]. eoxPL also are immunogenic in vivo, with elevated eoxPL-specific IgG detected in aPLS patients [39].

4. PAF, a plasmenylcholine with potent signaling activities

PAF is a structurally unique plasmenylcholine (alkyl bond at *sn1*) with an acetyl group at the *sn2* position that signals at extremely low concentrations (around 10^{-14} M) via the PAF receptor, a GPCR, stimulating multiple signaling cascades (Supplementary Figure 3B).

The PAF receptor is expressed by numerous blood cells including neutrophils, monocytes, eosinophils, and platelets [64]. It triggers multiple biological actions, including aggregation, calcium mobilization, superoxide release, eicosanoid generation, chemotaxis, adhesion, and cytokine generation in multiple circulating cell types, including platelets, neutrophils, and eosinophils [65].

PAF is generated via both remodeling and de novo pathways [66, 67] (Supplementary Figure 3B). Remodeling is the prominent biological mechanism and involves replacing an acyl with an acetyl moiety at the *sn2* position. De novo generation involves acetylation, dephosphorylation, and PC addition to a lysoPA, with this last step thought to be the source of endogenous PAF for homeostatic functions [67].

Two enzymes are involved in dynamically modulating PAF levels in the circulation. An acetyl transferase generated by circulating neutrophils and monocytes metabolizes lyso-PAF to PAF through addition of an acetyl group at the *sn2* position. This is the prominent source of PAF during inflammation and allergies [68]. On the other hand, PAF is removed by the action of PAF acetyl hydrolase (PAF-AH), a circulating enzyme generated by multiple cell types, including innate immune blood cells. Platelets are also a source of PAF that is generated during aggregation.

Several studies have evaluated the potential for circulating PAF to act as a biomarker/predictor of cardiovascular disease or events. Despite its association with higher disease burden, PAF-AH itself may represent a target for cardiovascular prevention, since PAF metabolism can lead to generation of pro-inflammatory lipids, such as lysoPC and lysoPAF. This concept led to extensive evaluation of reversible PAF-AH inhibitors, such as darapladib, in trials; however, these yielded mixed results, lacking sufficient affect on primary endpoints [24].

5. PLC in innate immune signaling.

PLCs are a class of enzymes that cleave PLs on the glyceride side of the phosphodiester bond, leading to diacylglycerol (DAG) formation and release of the phosphorylated PL headgroup (Figure 3A). PI-specific PLCs are the most important PL in immune cells, comprising approximately thirteen enzymes represented by six isotypes that play key roles in signal transduction [69]. PI-PLC hydrolyses PI-4,5-bisphosphate (PIP2) to form DAG and Ins(1,4,5)P3, which in turn can activate PKC and mobilize intracellular calcium, respectively to regulate both acute and chronic responses in immune cells. PLCs are activated by GPCRs and tyrosine kinase-linked receptors (RTKs) in platelets and leukocytes. RTK-activated PLCγ subtypes are the most important in these cells, playing key roles in supporting neutrophil respiratory burst, phagocytosis, adhesion, and cell migration. PLCγ2 is important for hemostasis: mice lacking this isoform exhibit a bleeding defect, with glycoprotein VI- and CLEC-2-dependent platelet responses abolished [70-74]. This isoform also plays a role in platelet-dependent clot retraction [75]. Mast cells, NK cells, and murine neutrophils express both PLCγ1 and PLCγ2 isoforms [76-79]. In all these cells, these PLCγ isoforms modulate TLR signaling through multiple mechanisms (reviewed in [80]). In addition, several GPCR-activated PLCβ isoforms have been detected in immune cells, with these playing key roles in the differentiation and activation of both innate and adaptive immune responses, with increases in free calcium and PKC activity being essential [69, 81]. Recently, PLCδ was shown to act as a negative regulator of macrophage-mediated phagocytosis [82].

6. PLD signaling in innate immune cells.

PLD enzymes hydrolyze PC, the most abundant external-facing PL, generating phosphatidic acid (PA) and choline (Figure 3A). They are activated in response to several GPCRs and RTKs (reviewed in: [83]). PA has a number of critical functions in innate immune signaling. With a small, negatively-charged headgroup, PA induces negative curvature thought to be important for production of vesicles and membrane fusion, thus playing a central role in membrane remodeling [84]. PA also acts as an intracellular signal, binding several cellular proteins including the GEFs DOCK2 and SOS, which activate Rac1 and Ras, whilst the PA-binding domain of cyclicAMP-PDE4A1 has been clearly defined[85-87]. Innate immune cells and platelets express the isomers PLD1 and PLD2, both known to play key roles in thrombotic disease [88-91]. *Pld1*$^{-/-}$ mice exhibit reduced $\alpha_{IIb}\beta_3$-dependent platelet activation, rendering them resistant to pathological hemostasis events such as strokes and pulmonary embolisms [92]. Studies in knockout mice and the use of isoform-specific inhibitors indicated that PLD1, but not PLD2 is required for neutrophil ROS production[93]. The concerted action of PLD and PLA_2 leads to formation of lysoPA, generated by thrombin-stimulated platelets, and is potentially able to stimulate aggregation itself via GPCR signaling [94] (Figure 6A); this is in addition to lysoPA generated by the action of autotaxin (lyso-PLD) upon lysoPC.

7. Phosphoinositides :

The seven phosphoinositides (PI(3)P, PI(4)P, PI(5)P, PI3,4P2, PI(4,5)P2, PI(3,5)P2 and PIP3) each demonstrate signaling capabilities (Figure 3B). The phosphoinositide 3-kinase (PI3K) enzymes catalyze hydroxyl group phosphorylation at the 3-position of the inositol headgroup of PI and its phosphorylated derivatives. The eight mammalian PI3K enzymes sort into three groups: Class I (PI3Kα, β, γ, δ), Class II (PI3KC2α, β, γ) and Class

III (vps34). Class I PI3Ks phosphorylate PIP2 to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). Class II enzymes can phosphorylate both PI or PI4P to generate PI3P or PI(3,4)P2 on endosomes and the plasma membrane. Finally, Class III enzyme solely phosphorylates PI, producing PI(3)P on endosomes, which also plays a key role in autophagy [95].

Class IA PI3Ks (PI3Kα and PI3Kβ) are ubiquitously expressed, whilst PI3Kδ is restricted to myeloid and lymphoid cells (reviewed in [96]). The Class IB isoform, PI3Kγ, is differentially expressed, but is most highly expressed in myeloid cells. Each Class IA PI3Kα, β, and δ comprises one of five homologous regulatory subunits (p85α, p85β, p50α, p55α or p55γ) complexed with a single p110 subunit, whereas PI3Kγ is made up of p110γ and either a p84 or p101 regulatory subunit. The soluble PI3Ks are recruited to the plasma membrane following receptor occupation. Antigen receptors, Fc receptors, integrins, cytokine receptors, and RTKs such as colony-stimulating factor (CSF) stimulate PI3Kα, β, and γ activity through tyrosine phosphorylation with the SH2 domains in the regulatory domain, binding to the phosphotyrosine residues. GPCRs such as fMLP activate PI3Kγ through G$\beta\gamma$ subunits binding to p84 or p101 and to p110γ. G$\beta\gamma$ has also been shown to bind to and thus activate p110β. Membrane association is further promoted by binding of the Ras-binding domain of the p110 subunits, associating with either Ras (p110α, δ, γ) or Rac (p110β).

PI3K activity generates PIP3, which binds to PH domains in target proteins and activates signaling cascades. The phosphorylation and activation of the serine kinase PKB (also known as AKT) is an ubiquitous response to PI3K activation that can activate TORC1 to promote cell growth, but also activates a metabolic shift, as in CTL activation [97]. PKB also phosphorylates and inhibits the nuclear localization of FOXO transcription factors critical in lymphocyte differentiation. PIP3 is dephosphorylated to regenerate PI(4,5)P2 by PTEN, but can also generate PI(3,4)P2 through SHIP1/2 activity [98]. PI(4,5)P2 also binds PH domain-containing proteins and, whilst many of these also bind PIP3, there is PI(3,4)P2 selectivity notably DAPP-1 [96]. Lipidomic analysis has shown that PIP3 is not a single molecular species, with C36:1, C36:2, C36:3, C36:4, C38:2, C38:3, C38:4 and C38:5 being detected in neutrophils and macrophages [99, 100]. This variety may enable further selectivity in binding of different PH-domain proteins and thus downstream signaling [100]

The field's understanding of the distinct roles of PI3K isoforms has benefited from generation of knockout mice and selective small molecule inhibitors. These approaches have provided evidence that PI3Kγ is critical for extravasation and migration of neutrophils, monocytes, and eosinophils to inflammatory sites, with additional PI3Kδ involvement possibly occurring through integrin-mediated activation. PI3Kβ and δ regulate immune cell spreading and activation by mediating cell surface attachment to extracellular matrix or pathogens, such as fungi, and both isoforms are necessary for a maximal ROS response [101]. In addition, PI3K activity stimulates Rac through PIP3 regulation of GEFs and GAPs, leading to regulation of the actin cytoskeleton.

The development, proliferation, and differentiation of B and T cells are stringently regulated by PI3K activity, which involves PIP3-regulated signaling, including control of FOXO/FoxP3. However, PIP3 also activates BTK, which phosphorylates and stimulates PLCγ-producing DAG and calcium signaling that are important for both B cell proliferation and cytokine synthesis [102]. There has been extensive development of PI3K inhibitors, with a number approved for clinical use [103], particularly PI3Kδ inhibitors to treat chronic lymphocytic leukemia [104]. The importance of the PI3K pathway in human immune disorders is further emphasized by the presence of activating mutations in p110δ in a number of primary immune deficiency patients, who suffer particularly from recurring respiratory tract infections [105]. In contrast, deficiency in PI3Kδ signaling decreases Treg cell number and increases neutrophil numbers, which can cause an increase in endotoxic shock-related mortality [106].

8. Phospholipids as ligands for innate-like T cell populations.

Most immunologists consider T cell immunity only in the context of peptide recognition when presented by MHC molecules [107]. However, the family of MHC class I-like antigen-presenting molecules termed CD1 is ideally suited for capturing and displaying lipids for T cell surveillance [2]. This evolutionarily-conserved CD1 antigen-presenting family is sub-divided into group 1 (CD1a, CD1b, and CD1c) and group 2 (CD1d) members. CD1 group 1 is absent in mice, thus our understanding of their role in immunity is less developed than CD1d, which is present in mice and humans [108, 109]. Each CD1 isoform has distinct atomic architectures [110] and cellular trafficking properties, thereby enabling broad array of lipid classes (including PLs) that can either represent foreign or self-antigens to be bound.

Distinct populations of T cells subsequently recognize these CD1-lipid complexes. Lipid-reactive T cells play key roles in protective immunity, cancer, autoimmunity, and allergies. The most well-characterized lipid-reactive T cells are the innate-like type I Natural Killer T cells (NKT cells), which are restricted to the CD1d molecule [2]. Type I NKT cells rapidly secrete high amounts of a range of cytokines upon activation and have shown broad therapeutic promise [109].

Lipid-reactive T cells can specifically respond to a range of CD1-presented lipids, including PLs, a major lipid component of mammalian membranes. Indeed, subsets of type I NKT cells have exhibited autoreactivity towards PI, PE, and plasmalogen lysoPE (p-lysoPE) [111, 112] [113]. While the physiological role of NKT autoreactivity towards common PLs remain unclear, reactivity towards certain lysoPLs, including lysoPC, are reported to be associated with inflammatory responses [114]. Interestingly, p-lysoPE is an ether-linked PL that is generated in the peroxisome and thought to play a role in the development of NKT cells [115]. Further, infection by Hepatitis B virus (HBV) alters the levels of lipids, including lysoPLs within hepatocytes, which subsequently results in NKT cell activation, thereby playing a role in protective immunity [116].

The functional properties, T cell receptor (TCR) repertoire, and antigen reactivity of type II NKT cells are distinct from type I NKT cells [1]. Type II NKT cells are also described as responsive to bacterially-derived PLs, including PI and PG from *M. tuberculosis*, suggesting a role in protective immunity towards this devastating pathogen [4]. PG from *Listeria monocytogenes* was also shown to potently activate a subset of type II NKT cells [3].

Members of CD1 group 1 also present PLs, and T cell reactivity towards certain pollen-derived PLs has been associated with allergies [117, 118]. Further, CD1a, which is expressed predominantly in the skin and naturally present skin-derived oils [119], is associated with contact hypersensitivities [120] as well as bee sting allergies due to phospholipase activity derived from bee venom-generating LPLs that cause an inflammatory skin response [121, 122]. Moreover, aberrant reactivity towards CD1a-presented PLs is also implicated in allergies towards house dust mites [123]. CD1b-mediated autoreactivity towards rare mammalian PLs, including PG, have also recently been described and implicated in some autoimmune conditions [124] [125].

The conundrum of how lipid-reactive T cells distinguish between rare and common PLs was recently determined to arise from the ideal shape and charge complementarity between the TCR and CD1b-PG complex, whereas the polar headgroups of more common PLs are sterically-disfavored from binding to the TCR [5]. Further, T cell targeting of specific PLs, including methyl-lysophosphatidic acid (mLPA), when presented by CD1c+ leukemic cells is considered to play a role in anti-tumor immunity and hence may represent a likely candidate for novel immunotherapeutic approaches [6]. Collectively, T cell reactivity towards CD1-restricted PLs is emerging as a component of protective and aberrant immunity, although the breadth of reactivity towards distinct PLs and their role in physiology and pathophysiology remains germinal.

9. Summary

Herein, we summarized the major PL-dependent signaling pathways in circulating innate immune cells and their recognition by specialized populations of innate T cells during challenge. The importance of PLs in the acute response to injury extends far beyond their structural role, as they provide a major repository for activation-dependent signaling molecules such as PGs, oxylipins, and PAF during acute injury and inflammation and through T cell recognition, representing a way for the body to sense danger and appropriately respond during challenge. Importantly, PL-dependent signaling is not only mediated through long-known soluble paracrine mediators (oxylipins such as prostaglandins), but also through changing membrane biophysics that alter protein binding and activation both on the outside and inside of cells (eoxPL, phosphoinositides). Many questions remain in the emerging study of PL signaling, particularly relating to how PLs dynamically change, the role(s) of CoA-dependent versus –independent recycling pathways in their metabolism, how tissues recognize endogenous changes and respond, and ultimately how to harness these processes to improve detection, diagnosis, and treatment of human disease associated with aberrant PL generation and signaling.

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Figure Legends

Figure 1. Structures of the major PL classes. Panel A. The five phospholipid classes are shown: PS: phosphatidylserine, PI: phosphatidylinositol, PG: phosphatidylglycerol, PE: phosphatidylethanolamine, PC: phosphatidylcholine. Panel B. Ether and plasmalogen PLs. Panels C,D. Structures of the common fatty acids in PLs.

Figure 2. PL cleavage and PL asymmetry in innate immune cells. Panel A. Phospholipases hydrolyze PLs and are named by the site of hydrolysis, as shown. Panel

B. Platelets externalize PE and PS on activation, providing a negatively charged surface that supports coagulation factor binding via calcium-dependent mechanisms.

Figure 3. PLC, PLD and Phosphoinositide pathways. Panel A. PLC and PLD generate lipid intermediates that promote innate immunity. Panel B. PI is phosphorylated/dephosphorylated by a series of known enzymes, generating a complex array of transient lipid molecular species which display potent bioactivity.

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Figure 1

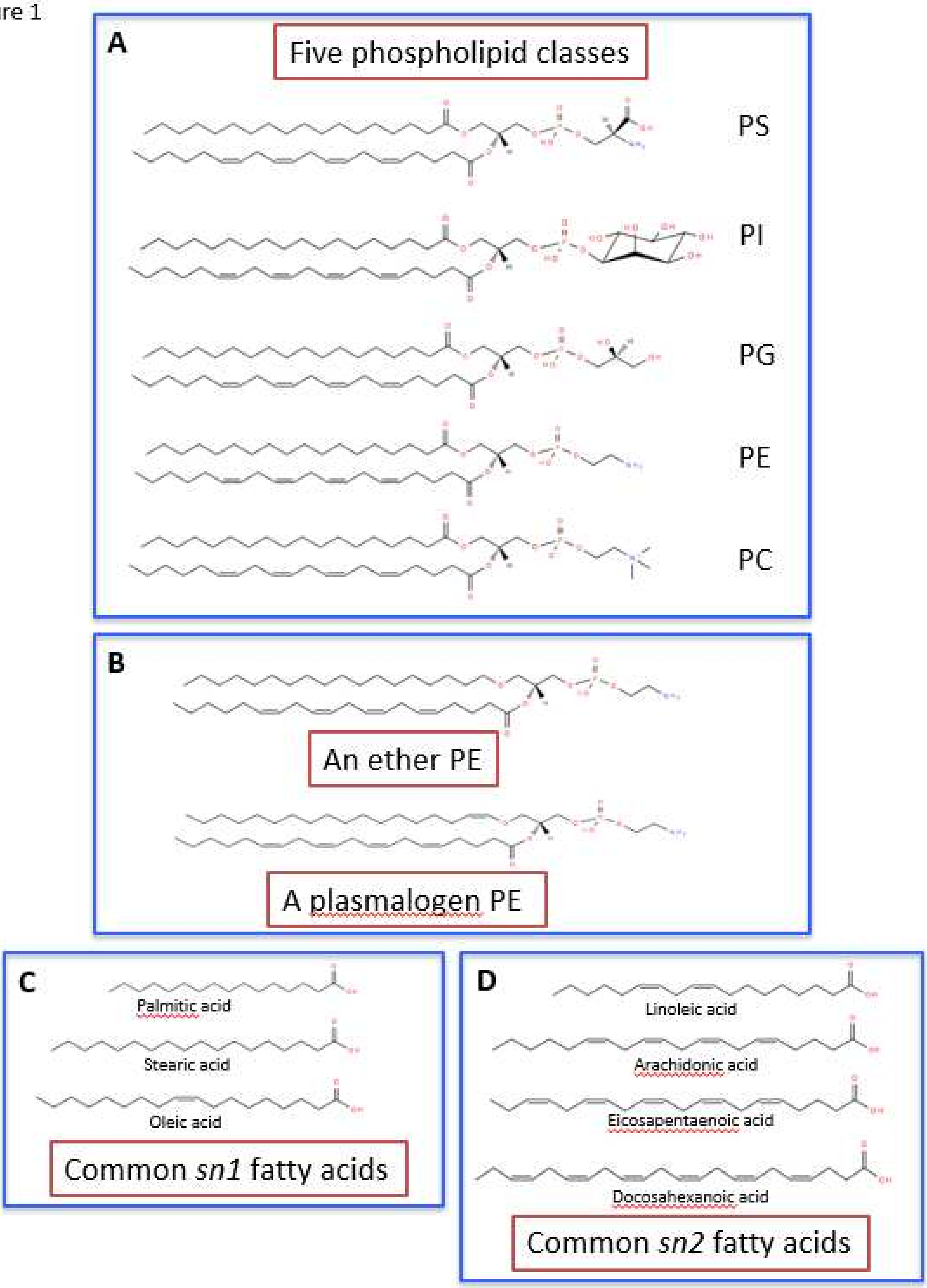

A
Five phospholipid classes
PS
PI
PG
PE
PC
B
An ether PE
A plasmalogen PE
C
Palmitic acid
Stearic acid
Oleic acid
Common *sn1* fatty acids
D
Linoleic acid
Arachidonic acid
Eicosapentaenoic acid
Docosahexanoic acid
Common *sn2* fatty acids

Figure 2

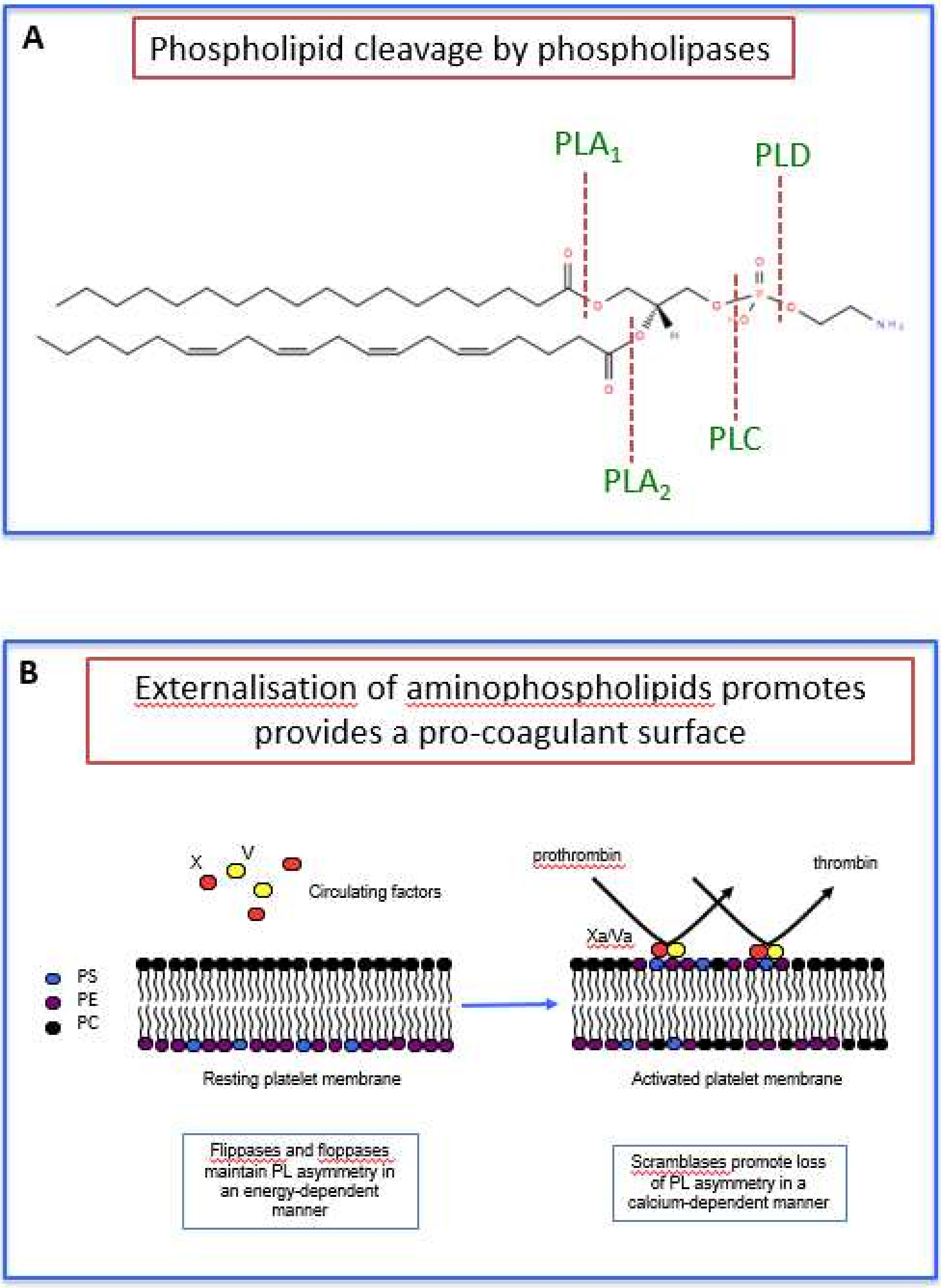
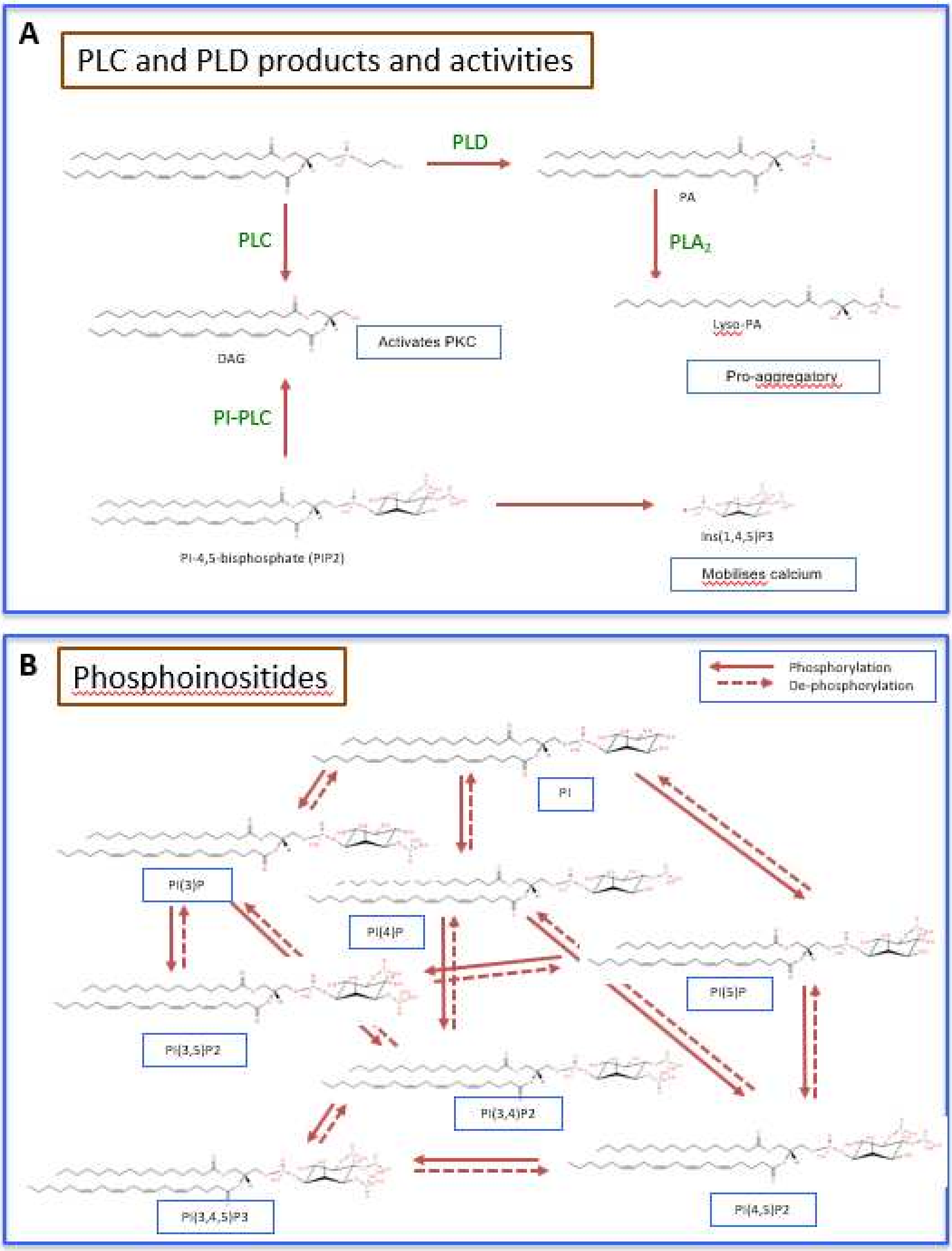
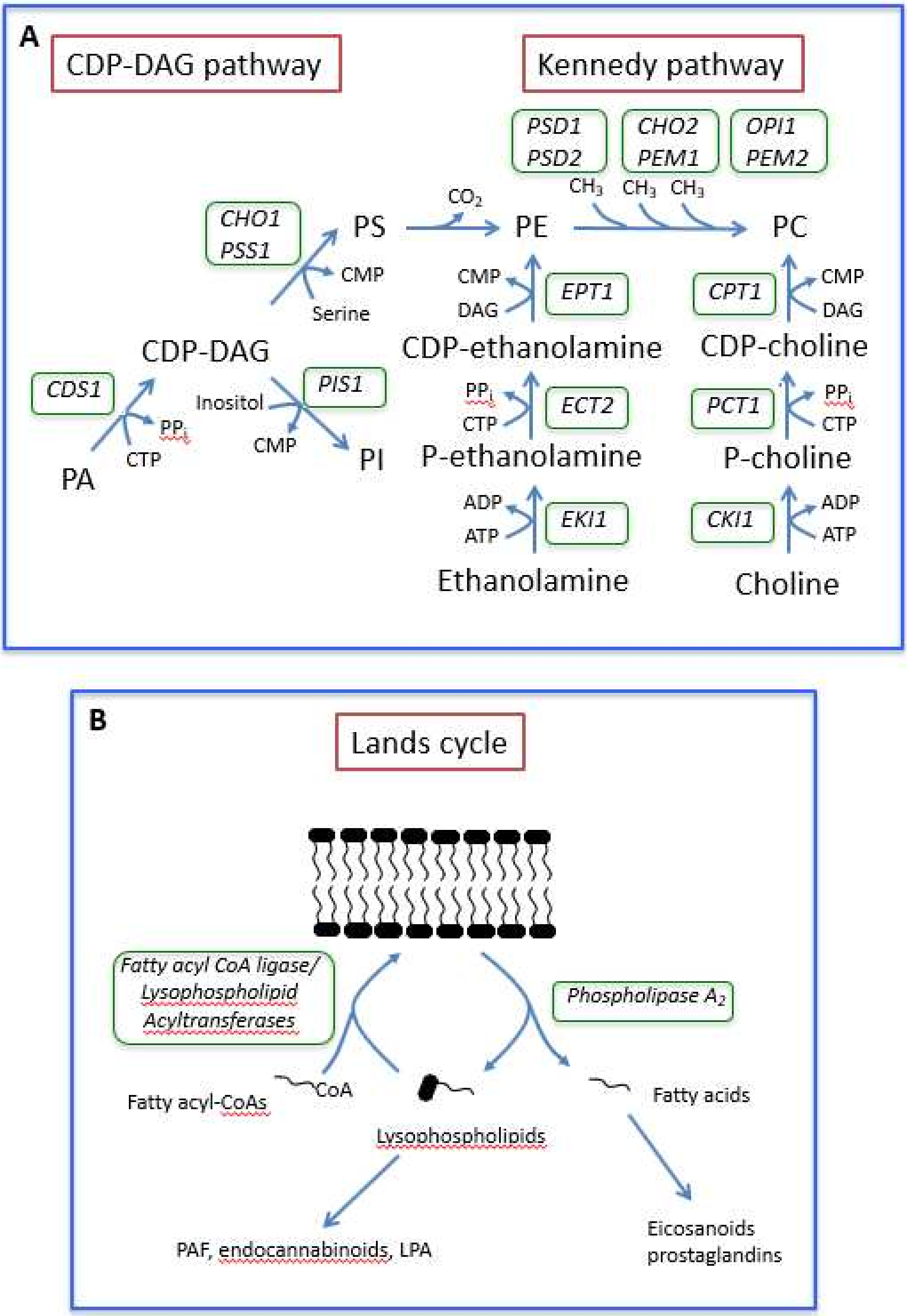

A
Phospholipid cleavage by phospholipases
PLA1
PLD
PLC
PLA2
B
Externalisation of aminophospholipids promotes provides a pro-coagulant surface
X
V
Circulating factors
prothrombin
thrombin
Xa/Va
PS
PE
PC
Resting platelet membrane
Activated platelet membrane
Flippases and floppases maintain PL asymmetry in an energy-dependent manner
Scramblases promote loss of PL asymmetry in a calcium-dependent manner

Figure 3

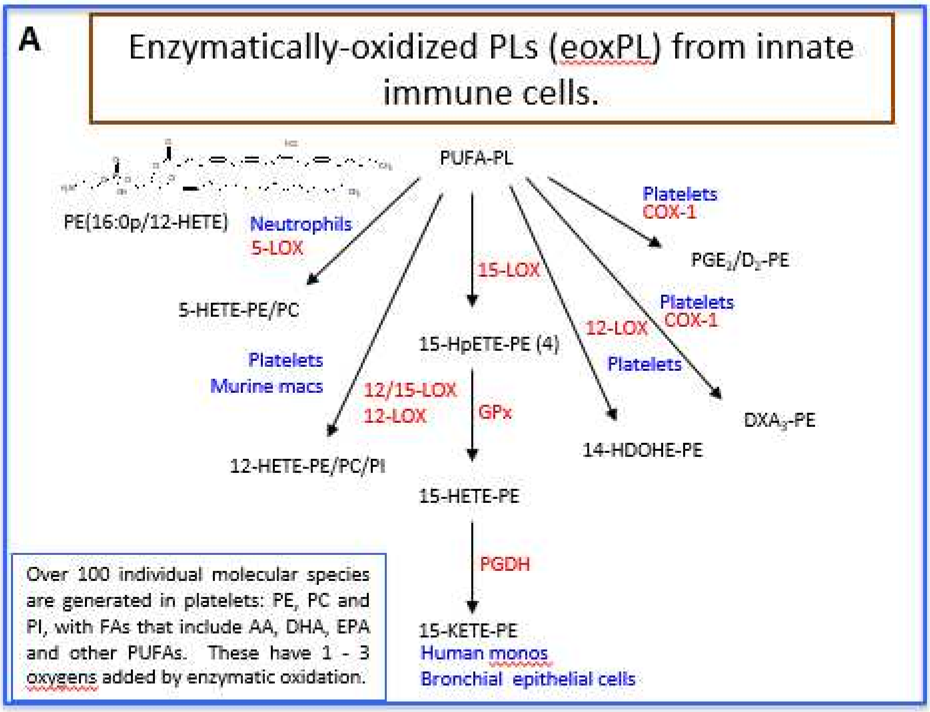

A
PLC and PLD products and activities
PLD
PA
PLC
PLA$_2$
Lyso-PA
DAG
Activates PKC
Pro-aggregatory
PI-PLC
PI-4,5-bisphosphate (PIP2)
Ins(1,4,5)P3
Mobilises calcium
B
Phosphoinositides
Phosphorylation
De-phosphorylation
PI
PI(3)P
PI(4)P
PI(5)P
PI(3,5)P2
PI(3,4)P2
PI(3,4,5)P3
PI(4,5)P2

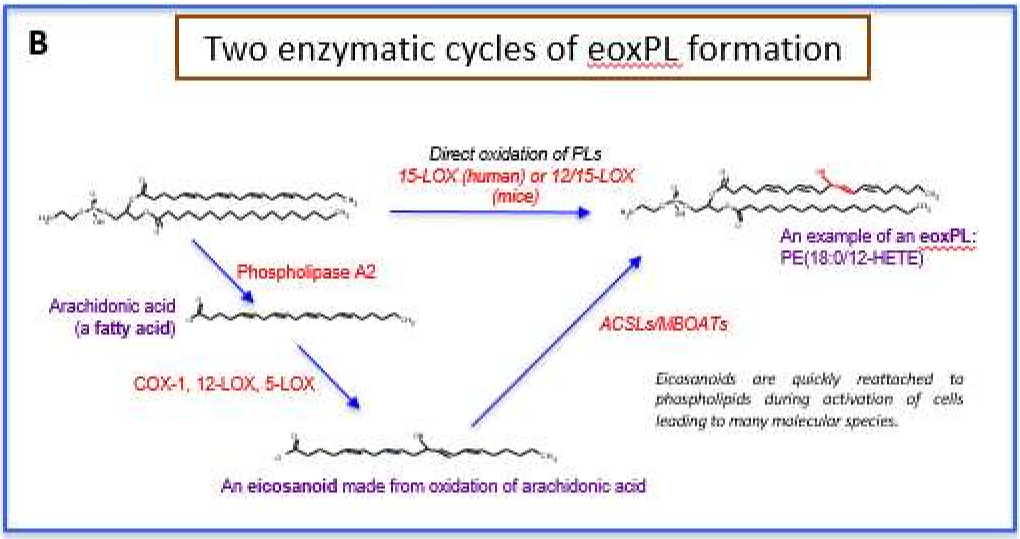
Supplementary Figure 1



Supplementary Figure 1. Pathways of PL generation and recycling. Panel A. CDP-DAG and Kennedy pathways. Gene names are indicated in italics. Panel B. Lands cycle. Hydrolysis and re-acylation of fatty acids into lysoPLs to generate phospholipids.

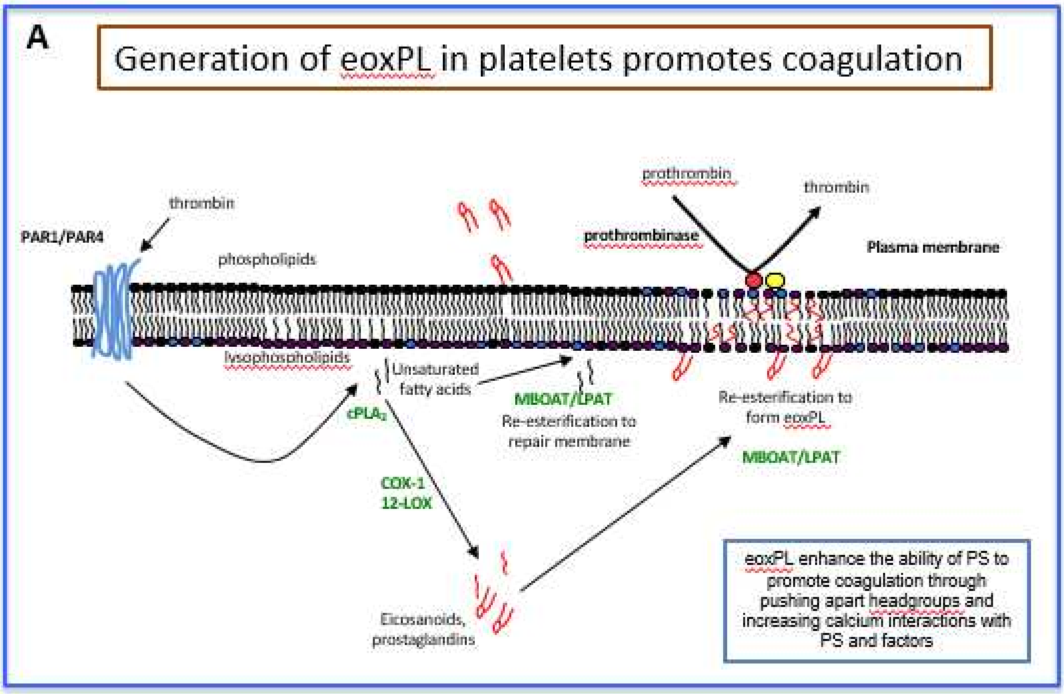
Supplementary Figure 2

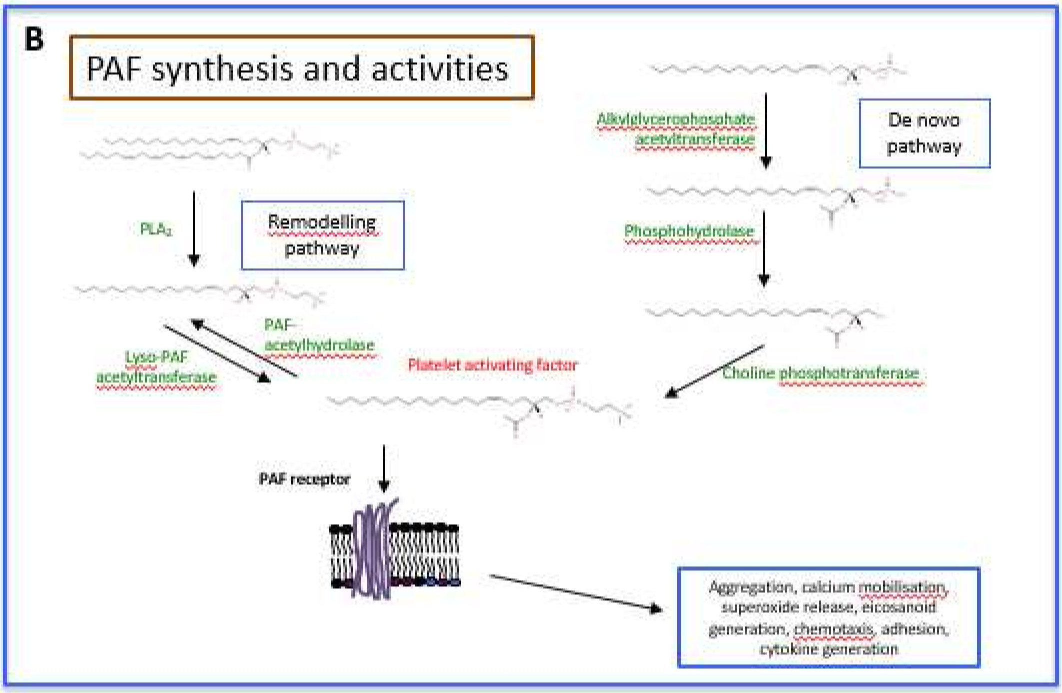




Supplementary Figure 2. Innate immune cells generate eoxPL via LOX or COX activities. Panel A. The diversity of eoxPL generated by immune cells. Panel B. Enzymatic cycles of eoxPL generation include direct oxidation and esterification of free acid eicosanoids into LPL, forming membrane-associated lipids.

Supplementary Figure 3





Supplementary Figure 3. Generation of eoxPL in platelets and the synthesis and activities of PAF. Panel A. eoxPL are generated in platelets following thrombin-mediated activation via a co-ordinated pathway including PLA2, COX-1/12-LOX, MBOAT/LPAT enzymes. Panel B. PAF is generated by two pathways and is potently bioactive through binding and activating the PAF receptor.