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REVIEW

The multifaceted role of PIP2 in leukocyte biology

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Abstract Phosphatidylinositol 4,5-bisphosphate (PIP2) represents about 1 % of plasma membrane phospholipids and behaves as a pleiotropic regulator of a striking number of fundamental cellular processes. In recent years, an increasing body of literature has highlighted an essential role of PIP2 in multiple aspects of leukocyte biology. In this emerging picture, PIP2 is envisaged as a signalling intermediate itself and as a membrane-bound regulator and a scaffold of proteins with specific PIP2 binding domains. Indeed PIP2 plays a key role in several functions. These include directional migration in neutrophils, integrin-dependent adhesion in T lymphocytes, phagocytosis in macrophages, lysosomes secretion and trafficking at immune synapse in cytolytic effectors and secretory cells, calcium signals and gene transcription in B lymphocytes, natural killer cells and mast cells. The coordination of these different aspects relies on the spatio-temporal organisation of distinct PIP2 pools, generated by the main PIP2 generating enzyme, phosphatidylinositol 4-phosphate 5-kinase (PIP5K). Three different isoforms of PIP5K, named α, β and γ, and different splice variants have been described in leukocyte populations. The isoform-specific coupling of specific isoforms of PIP5K to different families of activating receptors, including integrins, Fc receptors, toll-like receptors and chemokine receptors, is starting to be reported. Furthermore, PIP2 is turned over by multiple metabolising enzymes including phospholipase C (PLC) γ and phosphatidylinositol 3-kinase (PI3K) which, along with Rho family small G proteins, is widely involved in strategic functions within the immune system. The interplay between PIP2, lipid-modifying enzymes and small G protein-regulated signals is also discussed.

Keywords Phosphoinositide · Phosphatidylinositol 4-phosphate 5-kinase · Immune cell functions · Molecular signals · Cytoskeleton reorganisation

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Introduction

Phosphatidylinositol 4,5-bisphosphate (PIP2) is an inositol phospholipid located in the inner leaflet of the plasma membrane. PIP2 is the common substrate of two major lipid-modifying enzymes, namely phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K) (Fig. 1), and also plays an important role as a second messenger membrane-bound regulator and scaffold of proteins with specific PIP2 binding domains [1–3].

In the majority of cell types, PIP2 is mainly generated by type I phosphatidylinositol 4-phosphate 5-kinases (PIP5K), which mediates the phosphorylation of phosphatidylinositol 4-phosphate on the D5 position of the inositol ring (Fig. 1). Three PIP5K isoforms (α, β and γ) have been identified with differential subcellular localisations, thus providing both temporarily and spatially

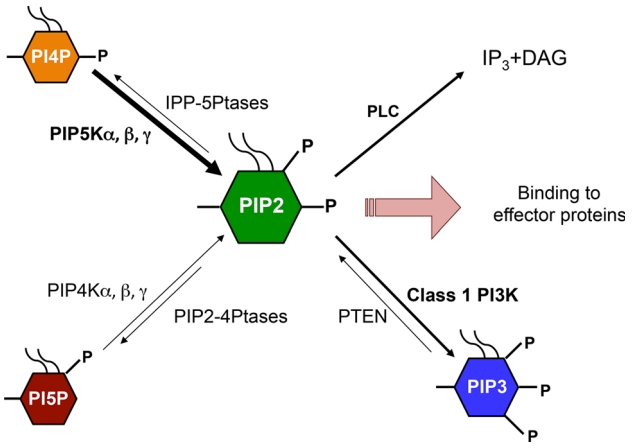

Fig. 1 Phosphatidylinositol 4,5-bisphosphate (PIP2) synthesis and turnover. PIP2 is mainly synthesised by type I phosphatidylinositol 4-phosphate 5-kinases (PIP5K) that phosphorylate phosphatidylinositol 4-phosphate (PI4P) and to a lesser extent by phosphatidylinositol 5-phosphate 4-kinases (PIP4K) that phosphorylate phosphatidylinositol 5-phosphate (PI5P). PIP2 may also derive from Phosphatase and tensin homolog (PTEN)-mediated PIP3 dephosphorylation Specific phosphatases, such as inositol polyphosphate 5-phosphatases (IPP-5Ptases) or PIP2-4 phosphatases (PIP2-4Ptases), dephosphorylate PIP2 yielding PI4P and PI5P, respectively. PIP2 may be either hydrolysed by phospholipase C (PLC) to generate inositol-1,4,5-trisphosphate (IP3) and diacylglicerol (DAG) second messengers or phosphorylated in the D3 position by class 1 phosphatidylinositol 3-kinase (PI3K) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3) that functions as a docking site for binding of signalling molecules. PIP2 may also function as a membrane-bound regulator of effector proteins

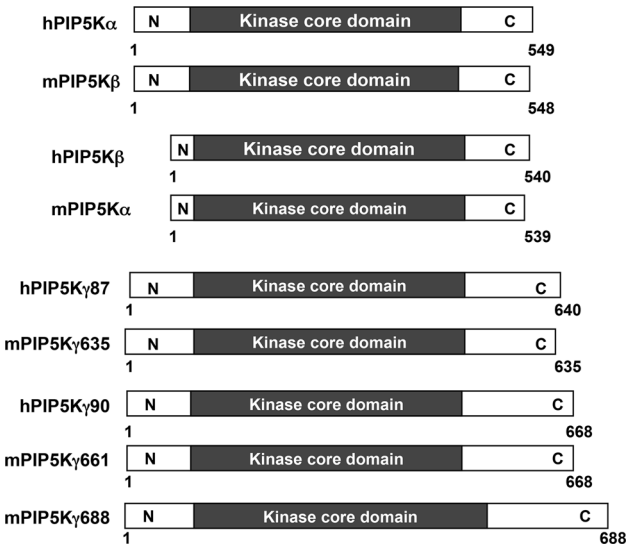

Fig. 2 Schematic representation of human (hPIP5K) and murine (mPIP5K) PIP5K isoforms with the conserved kinase core domain

regulated distinct pools of PIP2 [4–6]. It should be noted that the nomenclature for the human and murine PIP5Kα and β isoforms are switched. The three PIP5K isoforms display further variation in their sequence by alternative splicing. Eight PIP5Kα, two β and three γ splice variants have been described in mice, and three PIP5Kα, four β and one γ splice variants are present in humans. All PIP5K isoforms and splice variants share significant sequence identity throughout the kinase domain, whereas outside this region very little sequence conservation between the different isoforms is observed (Fig. 2). The catalytic domain of all PIP5K isoforms contains a subdomain, the activation loop, which determines substrate specificity and is critical for their activity and subcellular localisation. For instance, human PIP5Kα localises primarily to the plasma membrane [7–10], PIP5Kβ has been observed in nuclear and perinuclear vesicles [8] and PIP5Kγ was reported to localise to the intracellular membrane compartment [11] as well as to focal adhesion plaques [12]. In different cell types and in response to several receptors, PIP5Ks regulate several cellular functions including migration, focal adhesion, cell–cell adhesion and membrane cytoskeleton association, exocytosis and endocytosis, stress response, cytokinesis and nuclear processes [13]. It is becoming clear that the different isoforms play specific roles in different cell types and one PIP5K isoform may not compensate for the loss of another. More recently, a new concept in understanding PIP2 signalling has been highlighted. The discovery of a family of PIP2 binding proteins at the plasma membrane, such as myristoylated alanine-rich C-kinase substrate (MARCKS) [14], revealed that, in resting cells, a substantial fraction of PIP2 is sequestered and unavailable for binding to PIP2 effectors, while upon cell activation, it is released in response to changes of free Ca^{2+} levels.

Most of the proteins that regulate PIP5K activity belong to the Rho family of small GTPases, which are critical regulators of actin remodelling and vesicular trafficking [15]. Rho has been reported to interact directly with all PIP5K isoforms both in vitro and in vivo [16–18]. However, while the activation of PIP5Ks by Rho was dependent on GTP binding, the interaction between Rho and PIP5Ks was independent of GTP-loading [18], thus indicating that the activation of PIP5K by Rho could be indirect. This idea was supported by data from Weernink et al., who demonstrated that PIP5K activation by Rho was mediated by the Rho kinase ROCK [18, 19].

In addition to Rho, also Rac1 and Cdc42 have been reported to interact with all PIP5K isoforms and to activate them in a GTP-independent manner [7, 8, 20, 21]. Interestingly, the interaction of Rac1 with PIP5K has been associated to its ability to recruit PIP5Ks to the plasma membrane where PIP5Ks are activated [7, 21]. However, the isoform and the modality of PIP5K activation by Rac1 depend on the cell types and receptors. Besides Rho family, the Arf family small GTPase Arf6 has also been identified

as a main upstream regulator of PIP5K activity at the plasma membrane [22, 23]. In vitro and in vivo studies have demonstrated that membrane-bound Arf6 activates all PIP5K isoforms [24–26].

Independently of the cell types and/or of the small GTPases involved, it is clear from each of these studies that PIP5Ks and small GTPases strictly cooperate to regulate actin remodelling and actin-related cellular functions, such as exocytosis, endocytosis, adhesion and migration. One of the major regulators of PIP5Ks activity is phosphatidic acid (PA) [5, 27, 28]. PA may be generated through the hydrolysis of phosphatidylcholine by phospholipase D (PLD) or through diacylglicerol kinase (DAGK) in response to PIP2 hydrolysis by PLC. PLD2 has been found to interact and co-localise with PIP5Kα at the cell membrane [24, 29] and to stimulate cell adhesion through the activity of PIP5Kγ [30]. In addition, Arf6 also regulates PLD activity [31]; thus, Arf6 might activate both PLD and PIP5K to increase PA and PIP2. In addition to their lipid kinase activity, PIP5Ks have an intrinsic protein kinase activity and phosphorylate themselves on ser/thr residues. Autophosphorylation of all PIP5K isoforms occurs in vitro, is increased by PI and suppresses its lipid kinase activity [32]. Phosphorylation of PIP5Kβ at ser214 in the kinase homology domain by the cAMP-dependent protein kinase A (PKA) also leads to the inhibition of PIP5K activity [33]. Interestingly, dephosphorylation of PIP5Kγ at ser264, which is located within a PKA phosphorylation consensus site, stimulates its kinase activity [34]. PIP5Kγ contains other phosphorylation sites, such as tyr644 and ser645, which may regulate both PIP5K activity and the interaction with its binding partners. Ser645 phosphorylation by p35/Cdk5 and mitogen-activated protein kinase has been described as inhibiting PIP5Kγ interaction with talin and cell adhesion [35]. On the other hand, phosphorylation of the adjacent tyr644 by Src kinase activates PIP5Kγ [36]. By contrast, tyrosine phosphorylation of PIP5Kβ has been suggested to exert inhibitory effects [37]. Thus, depending on the isoform and cell type, PIP5K phosphorylation may have opposite effects.

One of the features of the immune system is the ability of cells to turn from a resting to an active state, relying on the engagement of an array of activating and inhibitory receptors. In this review, we illustrate the state of the art in understanding how PIP5Ks and PIP2 metabolism impacts on the innate and the adaptive immune cellular functions.

Mast cells

Mast cells are the main effectors in IgE-dependent inflammatory responses. Upon activation, they undergo acute and massive release of pro-inflammatory mediators stored in pre-formed secretory granules. Indeed, mast cells and mast cell lines have represented a paradigm for the study of regulated exocytosis in professional secreting cells.

The main route for mast cell activation is the high-affinity receptor for IgE, FcεRI, whose cross-linking by antigen initiates PLCγ-mediated Ca^{2+} mobilisation, an essential step for mast cell degranulation and cytokine production [38]. The activation of PLCγ results in the hydrolysis of PIP2 to generate the second messenger inositol-1,4,5-trisphosphate (IP3), which triggers the mobilisation of intracellular Ca^{2+} from endoplasmic reticulum (ER). Mast cells are the first model system whereby the calcium release-activated calcium (CRAC) channels [39] has been demonstrated [40]. Depletion of ER Ca^{2+} activates at plasma membrane the store-operated Ca^{2+} channels (SOCs), which represent the major route of calcium influx in immune cells. More recently, two key players of SOC entry have been identified: STIM1, an ER-localised calcium sensor [41, 42] and Orai1/CRACM1 [43, 44].

Initial study demonstrated that PIP2 and PA are critical components of exocytic machinery in mast cells and that Arf1 and Arf6 synergistically regulate PIPK5α activity [45]. Accordingly, by employing a quantitative immunofluorescence approach to follow PIP2 dynamics during mast cell exocytosis, Hammond et al. demonstrated that plasma membrane PIP2 becomes transiently depleted during degranulation. Such PIP2 consumption, due to PLC activation, was strictly necessary for exocytosis [46]. More recently, Vasudevan et al. provided evidences that PIP5Kβ and PIP5Kγ synthesise functionally different pools of PIP2 at the plasma membrane with distinct roles in antigen-stimulated IP3 production and SOC entry. The PIP5Kγ-dependent PIP2 pool positively regulates antigen-stimulated Ca^{2+} release from ER stores, whereas the PIP5Kβ-dependent pool negatively regulates SOC entry [47]. The existence of distinct PIP2 pools, which differently regulate calcium response, was confirmed by data from Calloway et al., demonstrating that the ratio of PIP2 that associates or not with detergent insoluble raft regulates the coupling of the ER sensor STIM1 with the Ca^{2+} channel protein ORAI1 during SOC entry [48].

Actin dynamics represent a primary aspect in degranulation responses. Indeed, filamentous (F)-actin behaves as a matrix required for myosin actin motors acting in vesicle transport to plasma membrane as well as a mechanical barrier preventing secretory vesicle docking and exocytosis of secretory granules.

The ubiquitously expressed neural Wiskott–Aldrich syndrome protein (N-WASP) and the hemopoietic WASP are key regulators of actin polymerisation leading to actin nucleation and branching through the actin-related protein

2/3 (Arp2/3) complex in response to upstream inputs [49, 50]. Seminal studies demonstrated that PIP2 activates WASP by binding to a short polybasic region involved in N-WASP auto-inhibition [51, 52].

In line with the finding that the majority of the requirement for PIP2 hydrolysis occurs downstream of Ca^{2+} signalling [46], Wollman et al. demonstrated that antigen activation of mast cells induces coordinated oscillation in Ca^{2+}, PIP2 and cortical actin levels. In live cells, the authors demonstrated that Ca^{2+} increases concomitantly with PIP2 reduction, preceding a drop in plasma membrane-localised WASP; the consequent reduction in F-actin levels is required to ensure the efficient secretory response [53]. Accordingly, the finding that PIP5Kα-deficient mice exhibit in vivo enhanced passive cutaneous and systemic anaphylaxis, due to an amplified degranulation and cytokine production after FcεRI cross-linking, also highlighted a negative correlation between PIP2 levels, actin polymerisation and mast cell degranulation. In this context, PIP5Kα behaves as a negative regulator of FcεRI-mediated cellular responses by increasing cortical actin and controlling FcεRI translocation to lipid rafts [54] (Fig. 3a).

Macrophages

A main function of macrophages is the capacity to engulf foreign agents, including microbes and apoptotic cells, in specialised compartments known as phagosomes, where microbial killing and digestion occur. Phagocytosis initiates with the interaction of ligands on target agents with specific receptors on the surface of macrophages. These include C-type lectin receptors, scavenger receptors, toll-like receptors (TLRs) and opsonin receptors, such as the Fcγ receptor (FcγR) and the complement receptor 3 (CR3). The former helps the interaction of macrophages with particles opsonised by IgG antibodies or by the complement molecule C3b, respectively. Upon particle binding, macrophages extend membrane processes (pseudopods) around the adherent particle until complete closure of the target particle in a phagosome. This process requires a series of spatially and temporally regulated steps, which are finely coordinated by the reorganisation of the actin cytoskeleton and the remodelling of the cellular membrane [55]. PIP2 is of particular importance for the assembly and remodelling of the F-actin structure during phagosome formation. At the onset of phagocytosis, PIP2 undergoes focal accumulation in the inner leaflet of the phagosomal cup, where it serves as a platform for the recruitment and activation of several proteins involved in actin polymerisation, such as profilin, cofilin, talin, vinculin, WASP and ezrin/moesin/radixin family members [56]. This localised increase of PIP2 is followed by a substantial decrease

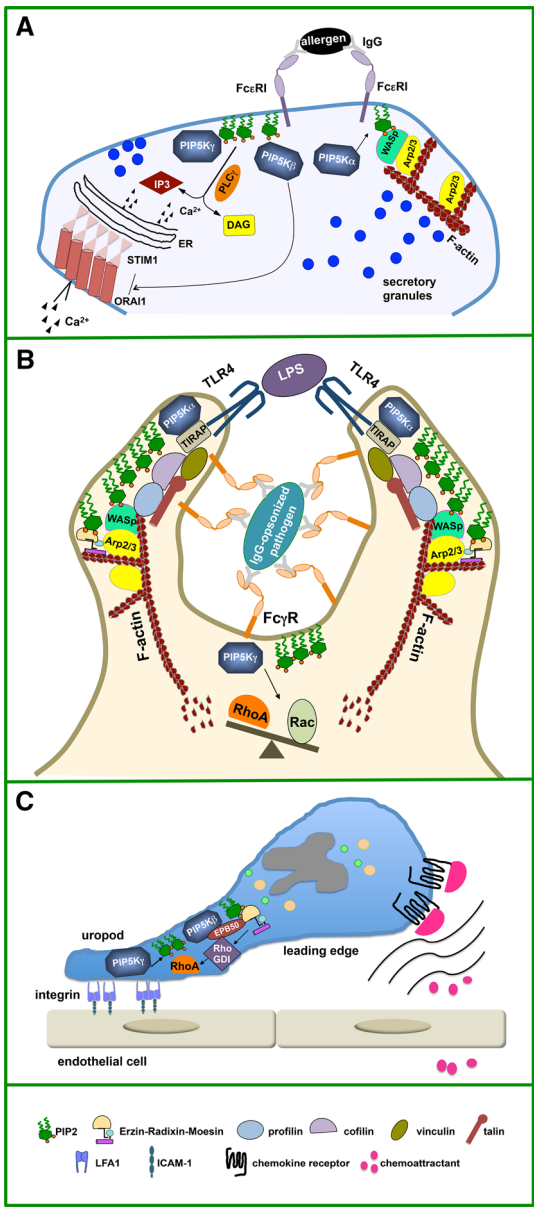

Fig. 3 Involvement of PIP5K isoform-dependent PIP2 pools in the regulation of the cytoskeleton reorganisation and signalling events. **a** Mast cell. PIP5K β and γ isoforms have opposite functions on FcεRI-induced Ca^{2+} response and granule release. **b** Macrophage. PIP5Kα and PIP5Kγ have distinct role in the regulation of FcγR-induced phagocytosis and TLR signalling. **c** Neutrophil. PIP5K isoforms control signals involved in neutrophil polarisation during cell migration and chemotaxis

during later stages of phagocytosis, thus allowing the actin depolymerisation processes essential for the closure and scission of the phagosome [57]. Several lines of evidence indicate that PIP5K isoforms are crucial for orchestrating actin remodelling at sequential stages of phagocytosis.

A first study by Coppolino et al. showed that the PIP5Kα isoform accumulated in the early stages of FcγRIIA-mediated phagocytosis, thus contributing to the transient

increase of PIP2 levels and of F-actin in the phagosomal cup without affecting the binding of phagocytic cells to the opsonised particles [20]. These data were also confirmed and expanded by Szymanska et al., who described the recruitment of PIP5Kα to detergent-resistant membrane rafts and its co-localisation with F-actin and PIP2 at the tips of filopodia, lamellipodium and ruffles, during FcγRIIA-induced phagocytosis. The displacement of PIP5Kα from the plasma membrane significantly diminished PIP2 levels and impaired formation of lamellae and filopodia, thus compromising both cell spreading and phagocytosis [58]. A more detailed study revealed that both PIP5Kα and PIP5Kγ were involved in FcγR-induced phagocytosis. Indeed, Mao et al. evidenced that bone-marrow-derived macrophages from PIP5Kγ knockout mice displayed high actin polymerisation but defects in phagocytosis. Closer analysis of these events revealed that both PIP5Kα and PIP5Kγ were recruited to the phagocytic cup, where they controlled distinct sequential steps. PIP5Kγ was essential for the initial phase of attachment of FcγRIIA to IgG-opsonised particles and FcγRIIA microclustering. PIP5Kγ also alters the balance of the activation states between Rac and RhoA, thus allowing actin depolymerisation. Conversely, PIP5Kα$^{-/-}$ BMM exhibited no defects on the adhesion of FcγR to IgG-opsonised particles, but displayed impaired WASP and Arp2/3 activation and actin accumulation at the phagocytic cup [59]. Similar results were obtained by analysing the role of Arf6. The overexpression of both constitutively active and dominant-negative forms of Arf6 impaired FcγR-promoted uptake and phagocytosis [60, 61] by interfering with both membrane recruitment and recycling, as well as with pseudopod extension [62].

In addition to controlling phagocytic pathways, PIP5K activity was also involved in regulating TLR signalling. TLRs function as primary sensors of microbial infections, recognising microbial molecular patterns ranging from bacterial-cell surface components to viral genomes, thus triggering both innate and adaptive immune responses [63, 64]. Involvement of PIP5Ks in the regulation of TLR4, a receptor of bacterial lipopolysaccharide (LPS) [65], was initially suggested by observations from Kagan and Medzhitov that the binding of a critical signalling mediator of TLR4 [66], TIRAP, to PIP2 was essential for its recruitment to the plasma membrane and that Arf6 played a critical role in this process [67]. A functional role of PIP5Kα in TLR4-mediated activation of macrophages was also suggested by Lee et al., who found that LPS stimulation of microglia increased both PIP2 levels and PIP5Kα expression [68]. The same group confirmed these initial observations by evidencing that a dominant-negative mutant of PIP5Kα impaired PIP2 production and the membrane recruitment of TIRAP in LPS-stimulated microglia. As a result, TLR4-associated signalling pathways and pro-inflammatory mediator production were both strongly inhibited [69]. Based on these observations, a role for PIP5K in regulating critical functions of macrophages, ranging from the ingestion and elimination of pathogens to the signals regulating the expression of genes involved in host defence from infection, can be envisaged (Fig. 3b).

Neutrophils

Neutrophils are key players in acute inflammatory responses contributing to both host defence and inflammation-related tissue injuries. Neutrophils are the most motile cells in higher organisms and have represented a model system for the study of leukocyte migration. During the inflammatory process, neutrophils extravasate across the vessel wall through a multistep process based on the rolling on and subsequent firm adhesion to endothelial cells followed by transmigration through the endothelium. Hence, they migrate to the sites of injury and infection in response to chemoattractant gradients. Directional cell movement in response to external chemical gradients relies on a spatial and functional asymmetry resulting from front-rear polarity: an up-gradient protrusive leading edge, where Rac-induced F-actin polymerisation occurs, and a down-gradient retroactive tail (uropod in leukocytes), where RhoA-mediated actomyosin contraction takes place [70, 71].

PIP2 and PIP5K isoforms have been described as key participants in the integration of front-rear signalling and in the maintenance of phosphoinositide asymmetry during cell migration. PI3K represents the hallmark of the leading edge signals in polarised neutrophils [72, 73]. Accordingly, in chemoattractant-stimulated neutrophil like HL-60 cells, PIP5Kα and γ along with PIP2 accumulate at the leading edge [74], likely ensuring proper levels of PI3K substrate. In mouse bone-marrow-derived neutrophils, a role for PIP5Kγ in determining initial cell polarity and directional responses has been clearly demonstrated in vivo. PIP5Kγ has two major splicing variants: a short 87 kDa protein (PIP5K1C-87) and a longer one with 28 additional amino acids at its C-terminus (PIP5KIC-90). Xu et al. demonstrated that integrin ligation by itself induces polarisation of PIP5K1C-90, which in turn promotes the polarised activation of RhoA at uropod in response to chemoattractants, thus allowing neutrophil adhesion to endothelial cells. RhoA polarisation at uropod helps evade its suppressive effect on lamellipodia formation, which is required for neutrophil extravasation [75].

Similar data were obtained by Lokuta et al., who evidenced a role of the PIPK5γ661 splice variant (PIP5K1C-90) as a component of the backness signal regulating cell

retraction during chemotaxis. They demonstrated that PIPK5γ661 and PIP2 are enriched in the uropod during chemotaxis of both primary murine neutrophils and neutrophil-differentiated HL60 cells [76].

Lacalle et al. showed that human PIP5Kβ is polarised at the uropod of chemotacting neutrophil-differentiated HL60 cells [77]. The PIP5Kβ C-terminus interacted with EBP50 (4.1-ezrin-radixin-moesin (ERM)-binding phosphoprotein 50), thus enabling interaction with ERM proteins, the activities of which are regulated by PIP2 binding and the Rho-GDP dissociation inhibitor (RhoGDI) [78]. As ERM proteins inhibit RhoGDI leading to RhoA activation [79], localised PIP2 production at the cell rear controls cell contractility (Fig. 3c). In support of this possibility, siRNA-mediated PIP5Kβ gene silencing inhibited cell polarisation and impaired cell directionality during chemotaxis [77].

Natural killer cells

Natural killer (NK) cells are innate immunity lymphocytes operating at the interface between innate and adaptive immunity. They directly contribute to immune defences through their effector functions, namely cytotoxicity and cytokine secretion, and indirectly by regulating antigen presenting cell (APC) and T cell responses. Together with cytotoxic T lymphocytes (CTLs), NK cells are major actors in immune protection against viral infections and cell transformation [80, 81]. Activation of NK cells is regulated by the balance between activating and inhibitory signals generated through a multitude of germ-line encoded receptors following the recognition of ligands expressed on the surface of target cells [82]. Killing of target cells occurs as a result of the polarised secretion of cytotoxic mediators, such as perforin and granzymes, stored in specialised lysosomes termed lytic granules. This process is finely regulated. It involves several steps, including the formation of a cytolytic synapse and the rapid reorientation of the microtubule-organising centre and lytic granules towards the target contact area, followed by granule docking, priming and fusion at specialised secretory domains [83, 84].

The activation of phosphoinositide metabolism is a critical step in the signalling pathways leading to activation of cytolytic machinery [85]. Indeed, in primary human NK cells expressing the PIP2-specific pleckstrin homology (PH) domain of PLCδ1 fused to green fluorescent protein (GFP) probe, the rapid consumption of a pre-existing PIP2 pool has been evidenced by time-lapse video microscopy during cytolytic interaction [86]. PIP5Kα and γ isoforms are mainly responsible for PIP2 synthesis in NK cells. The present authors have demonstrated that FcγRIIIA/CD16, the prototype of activating receptors, is coupled to PIP5Kα activity, while PIP5Kγ resulted constitutively active (R. Galandrini, personal communication). Indeed, CD16 stimulation rapidly induces PIP5Kα membrane recruitment and activation in an Arf6-dependent and Rho family GTPase-independent manner. Accordingly, expression of a dominant-negative Arf6 mutant reduces lytic granule exocytosis and PLD-dependent PA levels downstream to CD16 [87].

Actin rearrangement occurs at an early stage during the assembly of cytolytic synapses. As shown by 3-D confocal microscopy studies, actin rapidly polymerises at the synapse periphery of both CTLs and NK cells to arrange a dense ring of cortical F-actin surrounding a central area through which lytic granules are secreted [88, 89].

The leukocyte integrin LFA1 is critical for the generation of activating signals leading to polarised actin rearrangement. Downstream of LFA1, Rho family member Cdc42 becomes active [90]; its molecular effector WASP is directly responsible for actin polymerisation through activation of the actin nucleator Arp2/3 complex [49, 50]. Accordingly, in the absence of WASP, as in WAS immunodeficiency, or in the presence of actin inhibitors, both F-actin accumulation at the synapse and cytotoxic potential are reduced in both NK cells [91–93] and CTLs [94]. WASP activation critically depends on binding to PIP2 [51, 52], as evidenced in talin deficient mice [95]. The availability of PIP2 is crucial for relieving the intramolecular auto-inhibition of talin, thus promoting its binding to the β1-integrin tail [96]. In NK cells, talin binds to the cytoplasmic tail of β2-integrin and mediates the recruitment of Arp2/3, which initiates actin polymerisation upon LFA1 ligation [95]. Talin also associates with PIP5K, resulting in a localised increase in PIP2, which in turn cooperates in WASP recruitment and activation to the site of LFA1 ligation, thus promoting a polarised Arp2/3-mediated actin polymerisation.

Strict Ca^{2+}-dependence remains the hallmark of the lytic granule secretory phase [97]. Indeed, the essential role of PLCγ2 in granule exocytosis has been demonstrated in knockout mice [98]. Thus, patients with a mutation in either STIM1 or ORAI1 exhibit a defect in secretion, whereas lytic granule polarisation is unaffected [99].

Using shRNA-driven gene silencing, Micucci et al. demonstrated that PIP5Kα and γ are required for IP3 production by 2B4 activating receptor, indicating that both enzymes are needed to ensure the PIP2 levels required for proper PLCγ activity and lytic granule secretion. By contrast, PIP5Ks isoenzymes behave redundantly in the control of PI3K-dependent pathway and granule polarisation [86].

The Ca^{2+}-dependent factors required for lytic granule exocytosis are largely unknown. The high-affinity Ca^{2+} binding protein synaptotagmin is one possible candidate;

indeed, synaptotagmin VII has been implicated in exocytosis of lytic granules [100]. Furthermore, PIP2 itself is required for the ATP-dependent fusion phase of exocytosis, via a direct binding to the C2B domain of synaptotagmin [101], further contributing to its localisation and activation.

NK cells are endowed with the ability to execute multiple killing cycles in a short time period [102–104]. Such serial killing potential is thought to depend on release of a fraction of lytic granules and on retrieval of cytolytic machinery components [105]. Indeed, bidirectional trafficking of lytic granule proteins exposed at the plasma membrane on degranulation has been demonstrated [106, 107]. Molecular signals controlling endocytic traffic at cytolytic synapse are starting to be clarified. At the plasma membrane, PIP2 has been clearly distinguished in the regulation of endocytosis. Clathrin adaptors, such as the AP2 complex, recruitment of which is dependent on PIP2, regulate recruitment of clathrin to the plasma membrane at sites of endocytosis [108]. There is also a direct interaction between PIP5Kγ and the AP2 complex [109]. Recent findings from Capuano et al. report that a constitutive PIP5Kγ-dependent PIP2 pool is involved in the control of Munc13-4 re-internalisation through a clathrin/AP2-dependent endocytic route, which is functional to ensure the full serial killing potential in NK cells [107]. Such findings strengthen the analogy between neuronal and cytolytic synapse, where PIP5Kγ also triggers clathrin-mediated retrieval of synaptic vesicles [110].

At the final stage of endocytosis, PIP2 also recruits and activates dynamin GTPase, to complete the fission and release of endocytic vesicle from the plasma membrane [111] (Fig. 4a). Dynamin 2, which has been shown to be required for cytotoxicity in NK cells [112], may represent an additional effector molecule involved in granule recapture during exocytosis.

T lymphocytes

T lymphocytes are pivotal cell types from the adaptive immune system that play a critical role in cell-mediated immune responses. T cell activation requires the interaction of the T cell receptor (TCR) with a specific antigenic peptide presented by major histocompatibility complex (MHC) expressed on the surface of APCs [113]. This event induces the rearrangement of TCR, co-stimulatory receptors and ligands into a highly organised immunological synapse (IS) where many molecules co-localise to trigger important signalling pathways for T cell activation and effector functions [114]. One key role of PIP2 is to regulate TCR signalling by serving as a substrate for the generation of second messengers. TCR stimulation induces the activation of PLCγ1 that hydrolyses PIP2 into DAG and IP3

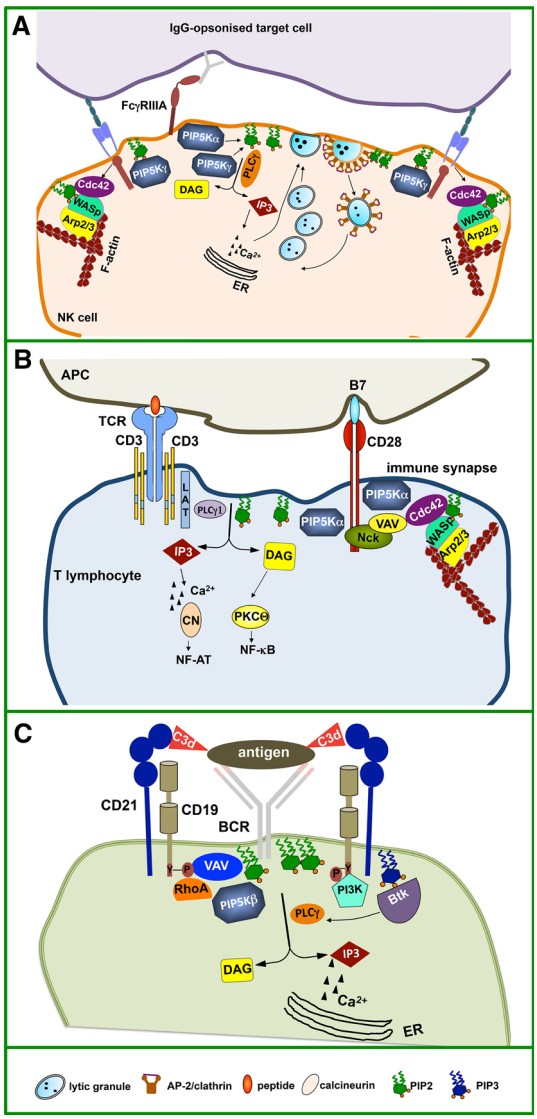

Fig. 4 Involvement of PIP5K isoform-dependent PIP2 pools in the regulation of the cytoskeleton reorganisation and signalling events. **a** NK cell. PIP5K α and γ isoforms control FcγRIIIA-dependent Ca^{2+} response and lytic granule exocytosis. PIP5Kγ regulates integrin-mediated adhesion and recycling of lytic granule components. **b** T lymphocyte. CD28 recruits both Vav1/PIP5Kα complexes and PI3K at the T:APC interface. PIP5Kα-dependent PIP2 pool serves as substrate for both PI3K and PLCγ1. PI3K generates PIP3 that stabilises membrane localisation of Vav1 and Nck/WASp/Arp2-3 complexes and promotes actin polymerisation. PLCγ1 hydrolyses PIP2 into DAG and IP3, thus inducing the activation of PKCθ and triggering the Ca^{2+} influx necessary for the activation of NF-AT and NF-κB transcription factors, respectively. **c** B lymphocyte. CD19 recruits Vav1, PIP5K and PI3K. Vav1-mediated recruitment of PIP5K to CD19 increases the local levels of PIP2, thus, favouring both PI3K and Btk-dependent PLCγ2 signalling pathways

(Fig. 1). The lipid DAG remains in the cellular membrane where it regulates recruitment and activation of protein kinase C (PKC) θ [115, 116]. By triggering Ca^{2+} response, soluble IP3 leads to activation and nuclear translocation of the nuclear factor of activated T cells (NF-AT) and the

expression of genes important for T cell activation [117]. PIP2 serves also as a substrate of class I PI3K which, by phosphorylating PIP2 on carbon atom 3, generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) [118] (Fig. 1). PIP3 binds to PH domains of several signalling molecules involved in the activation of TCR signalling cascades [119], including Tec-family tyrosine kinases (Itk) [120], protein kinase B (also known as Akt) [121], PLCγ1 [122], Vav1 and Vav2. However, TCR stimulation alone is not sufficient to activate these pivotal signalling pathways and requires the co-engagement of CD28 co-stimulatory molecule.

CD28 extra-cellular Ig-like domains bind to the cognate ligands, B7.1/CD80 or B7.2/CD86 on the surface of professional APCs, including macrophages, dendritic cells and activated B lymphocytes. Initial studies evidenced that CD28 facilitates early signalling events controlled by the TCR by lowering the threshold of the number of triggered TCRs [123], favouring the formation of large aggregates of lipid rafts [124] and thus enhancing tyrosine phosphorylation of TCR-controlled signalling effectors [125] and the downstream signalling pathways [126]. Another critical contribution of CD28 to TCR signalling is the activation of PI3K [127]. The cytoplasmic tail of CD28 contains the YMNM motif that binds the p85 subunit of class 1A PI3K, which in turn recruits the p110 catalytic subunit, leading to PI3K activation and PIP2 phosphorylation [128] independently of TCR stimulation [127]. Since PIP2 represents less than 1 % of plasma membrane phospholipids, new PIP2 must be synthesised to provide sufficient substrate in response to TCR and CD28 co-engagement [129].

Initial studies by Zaru et al. have evidenced that TCR engagement induces a rapid and sustained PIP2 turnover and CD28 co-stimulation strongly increases this process [130]. More recent data by Singleton et al. showed that PIP2 synthesis occurs at the T:APC interface and very early during antigen recognition it accumulates at the IS, where PIP2 is rapidly consumed by PLCγ1 and PI3K [131]. Spatio-temporal analysis of PIP2 synthesis in T lymphocytes suggested that different PIP5K isoforms play a differential role on the basis of their distinct localisation. PIP5Kβ and PIP5Kγ90 were mainly found at the distal pole which, during the early phases of T cell activation, was the site of slower PIP2 turnover. On the other hand, PIP5Kγ87 and PIP5Kα were enriched at the IS where intense PIP2 turnover occurs [132]. However, while PIP5Kγ87 recruitment at the T:APC interface was rapid and transient, interface accumulation of PIP5Kα was more pronounced and sustained, suggesting a major role of PIP5Kα in PIP2 refilling [132]. Recent data from Muscolini et al. confirm this hypothesis by demonstrating that CD28 co-stimulation regulates TCR-induced PIP2 synthesis by recruiting and activating PIP5Kα at the T:APC interface, thus sustaining CD28 co-stimulated Ca^{2+} influx, NF-AT nuclear translocation and IL-2 gene transcription [133].

In addition to functioning as a central substrate for second messenger generation, PIP2 is pivotal for the remodelling of the actin cytoskeleton necessary for the clustering of TCR and co-stimulatory receptors towards the T:APC interface. The formation of a stable T:APC conjugates is essential to allow the further clustering of TCR, co-stimulatory and signalling molecules at the IS. For example, T:APC contact for up to 24 h is required for optimal T cell proliferation and cytokine production [134–136]. The formation of a stable T:APC interaction requires the interaction between the integrin LFA1 on the surface of T cells with its ligand ICAM-1 on APCs. LFA1 activity is regulated by both a change of its affinity for ICAM-1 and by polarisation at the IS [137]. Data by Bolomini-Vittori et al. evidenced that PIP5Kγ selectively regulates the transition of LFA1 from a low intermediate to a high-activation state, by acting downstream of Rho and Rac-1 and favouring T cell arrest and stable adhesion [138]. In contrast, more recent data from Wernimont et al. show that $CD4^+$ T cells from PIP5Kγ90-deficient mice have increased LFA-1 adhesion to ICAM-1 and T:APC conjugate formation, as well as enhanced LFA-1 polarisation at the IS. As a result, increased proliferation and cytokine production in response to TCR and CD28 co-engagement were observed in PIP5Kγ90 knockout mice [139]. These data are consistent with the different localisations of the two PIP5Kγ isoforms during T:APC interaction, with the 87-kDa isoform transiently localised at the T:APC interface and PIP5Kγ90 in the uropod [132] where it may sequester key molecules mediating T:APC contacts. Thus, the apparent discrepancy between the two groups was explained by the selectivity of siRNA used by Wernimont et al. for silencing the PIP5Kγ isoforms, which specifically depleted the 87-kDa isoform but not the 90-kDa isoform [138].

Once LFA-1 has mediated a stable contact of T cells with APCs, sustained cytoskeleton rearrangement events occur for the relocalisation of receptors, lipid rafts and signalling complexes at the IS [140–144]. Interestingly, CD28 regulates the remodelling of the actin cytoskeleton independently of TCR [145, 146], thus delivering a unique signal necessary for both the initiation of TCR signalling as well as for CD28 autonomous functions [147, 148]. Muscolini et al. has recently highlighted that CD28 is the main regulator of PIP5Kα recruitment to the T:APC interface and that PIP5Kα activity is essential for CD28-mediated actin polymerisation. Vav1, a GEF for Rac1 and Cdc42 GTPases [149], was also identified as the linker molecule that couples the C-terminal proline-rich motif of CD28 to the recruitment and activation of PIP5Kα and Nck [150], a critical adaptor that cooperates with PIP5Ks in promoting N-WASp localisation and actin polymerisation [52]. PIP5Kα then synergises with PI3K, generating high local

concentration of PIP2 and PIP3 which, in turn, stabilises the membrane localisation of Vav1 and Nck/WASp/Arp2-3 complexes needed to promote actin polymerisation and activation of downstream signalling pathways (Fig. 4b).

B lymphocytes

B lymphocytes are cell types of the adaptive immune system that play a critical role in humoral immune response primarily by making antibodies against antigens. Signals initiated by the activation of B cell receptors (BCR) are crucial for B cell development, proliferation, differentiation and survival. Similarly to T cells, PIP2 is pivotal for B cell activation, by serving as a substrate for both PLCγ2 and PI3K [151, 152]. In B cells, PI3K and PLCγ2 are strictly connected by Btk, a member of the Tec-family tyrosine kinase that is fundamental for both BCR signalling and B cell development [136]. Indeed, mutations in Btk cause the B cell immunodeficiency X-linked agammaglobulinemia (XLA) in humans [153, 154]. Btk activation involves a two-step process: its tyrosine phosphorylation by Src family tyrosine kinases and its recruitment to the plasma membrane by binding PIP3 [136]. Thus, the dependence of Btk activation on PI3K activity together with its ability to stimulate PLCγ2 places Btk at a critical nexus in PIP2 metabolism. Thus, sustained local availability of PIP2 is crucial to guarantee sufficient substrate for both PI3K and PLCγ2. Thus, for instance, an association between Btk and PIP5Ks was demonstrated by Saito et al. who found constitutive PIP5K activity in Btk immunoprecipitated from murine splenic B cells [155]. More detailed analysis revealed that both PIP5Kα and PIP5Kβ associated with the Btk PH/tec-homology (TH) domain and co-localised with Btk in membrane rafts [156]. Similarly to TCR, efficient BCR signalling and B cell development and activation also require the activity of Rho/Rac GTPase and Vav [157, 158]. Data from Inabe et al. highlight a vital role for Vav3 in regulating PI3K function and sustaining PIP3 production and calcium mobilisation in B cells [159].

The activation of PI3K in B cells is mainly mediated by CD19, an essential co-receptor for the BCR that, through its association with the complement receptor CD21, positively regulates B cell responses [160, 161]. Similarly to CD28, CD19 contains two tyrosines, tyr482 and tyr513, within canonical binding sites (YxxM) for the src-homology (SH)2 domain of the p85α subunit of PI3K [160–162]. Interestingly, following stimulation CD19 also associates with the SH2 of Vav through phosphotyr391 [163, 164]. Vav recruitment and activation were also essential for CD19-mediated PIP5K activation and PIP2 synthesis [163]. The mechanisms by which Vav regulates CD19-mediated activation of PIP5Ks are at present unknown. These may involve Vav-dependent activation of RhoA, which has been found to associate with both PI4K and PIP5K activities and to regulate both PIP2 synthesis and BCR-dependent pathways [165]. However, data from O'Rourke et al. evidenced that PIP5K activation by CD19 and Vav was Rho-independent [163]. Alternatively, due to the strong similarity between CD28 and CD19, Vav may physically associate with PIP5K thus favouring its recruitment and activation by CD19 (Fig. 4c).

Conclusions

Stimulation of immune cells with different ligands triggers several signalling pathways involving PIP2: the regulation of both actin disassembly and polymerisation necessary for both endocytosis and exocytosis, as well as for the reorganisation of surface receptors and co-stimulatory molecules and the generation of cellular second messengers essential for gene expression and cell activation.

The accumulation of PIP2 at the site of receptor engagement is pivotal for directional orchestration of these processes. Thus, elucidation of the mechanisms whereby individual PIP5K isoforms fulfil distinct functions within the same cell is fundamental to increase knowledge of the mechanisms of leukocyte activation and function. A considerable challenge for the future will be to gain more in-depth understanding of the spatial and temporal coordination of molecular signals, which may account for different qualities of cell responses.

From a clinical perspective, PIP5Ks are interesting therapeutic targets by reason of regulating signalling pathways (i.e. PI3K, PKC and PLC) that are altered in tumours and in inflammatory as well as in autoimmune diseases. One selective PIP5Kα inhibitor has recently been identified and proved effective in inhibiting advanced prostate cancer progression [166] and T lymphocyte activation (L. Tuosto, personal communication). Thus, more extensive investigation of the mechanisms regulating PIP5K recruitment, activation and regulation by its interacting partners may prove useful for the design of more selective targeted therapies for immune-based diseases.

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