

# Molecular control of PtdIns(3,4,5)P3 signaling in neutrophils

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## Abstract

Neutrophils play critical roles in innate immunity and host defense. However, excessive neutrophil accumulation or hyper-responsiveness of neutrophils can be detrimental to the host system. Thus, the response of neutrophils to inflammatory stimuli needs to be tightly controlled. Many cellular processes in neutrophils are mediated by localized formation of an inositol phospholipid, phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3), at the plasma membrane. The PtdIns(3,4,5)P3 signaling pathway is negatively regulated by lipid phosphatases and inositol phosphates, which consequently play a critical role in controlling neutrophil function and would be expected to act as ideal therapeutic targets for enhancing or suppressing innate immune responses. Here, we comprehensively review current understanding about the action of lipid phosphatases and inositol phosphates in the control of neutrophil function in infection and inflammation.

**Keywords** innate immunity; inositol phosphate; lipid phosphatase; neutrophils; PtdIns(3,4,5)P3

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See the Glossary for abbreviations used in this article.

## Introduction

Neutrophils migrate from the blood to infected tissues in response to inflammatory stimuli, where they protect their host by engulfing, killing, and digesting invading bacterial and fungal pathogens [1–4]. Neutrophils sense invading pathogens in the extracellular environment via several classes of cell surface receptor: G-protein-coupled receptors (GPCRs) to sense chemoattractants, Fc or complement receptors for phagocytosis, adhesion molecules (such as selectins and integrins), cytokine receptors, and innate immune receptors (such as the Toll-like receptor; TLR). When these receptors are bound by their respective ligands, they activate diverse intracellular signal transduction networks that lead to chemotactic migration,

phagocytosis, degranulation, production of reactive oxygen species (ROS), and release of neutrophil extracellular traps [1,5].

Many cellular processes in neutrophils are mediated by localized formation of an inositol phospholipid, phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3), at the plasma membrane (Fig 1A). PtdIns(3,4,5)P3 is formed by the phosphoinositide 3-kinase (PI3K) family of enzymes, which transfer the terminal phosphate of adenosine triphosphate (ATP) to PtdIns(4,5)P2 at the D3 position of the inositol ring. PI3Ks are divided class I, II, and III enzymes, but only class I PI3Ks phosphorylate PtdIns(4,5)P2 to form PtdIns(3,4,5)P3 [6,7] (Fig 1B). PtdIns(3,4,5)P3 exerts its function by mediating translocation of various proteins via pleckstrin homology (PH) domains, which is a divergent protein module of approximately 120 amino acids found in many proteins involved in signal transduction [8] and that frequently mediates protein–protein or protein–phosphoinositide interactions. A subset of PH domains, including those in Btk, PKB/Akt, PLC- $\gamma$ , Gab1, PDK1, Grp1, ARAP3, and multiple Rho guanine nucleotide exchange factors (GEFs), drive membrane translocation of their host proteins via specific, high-affinity recognition of PtdIns(3,4,5)P3 [6,9–11]. This membrane translocation is crucial for PtdIns(3,4,5)P3-mediated cellular processes such as cell survival, proliferation, growth, differentiation, polarization, chemotaxis, cytoskeletal rearrangement, and membrane trafficking [12–15] (Fig 1C).

## Negative regulation of PtdIns(3,4,5)P3 signaling in neutrophils

Although neutrophil activation is essential for pathogen killing and clearance, excessive neutrophil accumulation or hyper-responsiveness of neutrophils can be detrimental to the host; the response of neutrophils to inflammatory stimuli therefore needs to be tightly controlled. Over 50 chemokines have been identified in humans, and many of them are constitutively present in blood or tissues. However, neutrophils will only migrate, generate superoxide, or degranulate in response to a strong and stable stimulus, which suggests that inhibitory factors must be present to suppress the positive signals elicited by a weak or unstable chemoattractant signals [16,17]. These intracellular inhibitors establish a threshold for neutrophil responses, and as a result, neutrophils only respond when they receive stimulation that overcomes the negative inhibitory effect. Here, we comprehensively

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**Glossary**

<b>5ptase IV</b>	phosphoinositide-specific inositol polyphosphate 5-phosphatase IV
<b>COPD</b>	chronic obstructive pulmonary disease
<b>CSE</b>	cigarette smoke extract
<b>G-CSF</b>	granulocyte colony-stimulating factor
<b>GEF</b>	guanine nucleotide exchange factors
<b>GPCR</b>	G-protein coupled receptor
<b>InsP3K</b>	inositol trisphosphate kinase
<b>InsP6K</b>	inositol hexakisphosphate kinase
<b>InsP7</b>	diphosphoinositol pentakisphosphate
<b>IPMK</b>	inositol phosphate multi-kinase
<b>PH-domains</b>	pleckstrin homolog domain
<b>PI3K</b>	phosphatidylinositol 3'-kinases
<b>PLC</b>	phosphatidylinositol-specific phospholipase C
<b>PtdIns(3,4,5)P3</b>	phosphatidylinositol 3, 4, 5 trisphosphate
<b>PtdIns(4,5)P2</b>	phosphatidylinositol 4,5-bisphosphate
<b>PTEN</b>	phosphatase and tensin homologue deleted on chromosome ten
<b>Rock</b>	RhoA-associated kinase
<b>ROS</b>	reactive oxygen species
<b>SHIP1</b>	SH2-containing inositol-5'-phosphatase-1
<b>TLR</b>	Toll-like receptor

review current understanding about the action of lipid phosphatases and inositol phosphates in the control of neutrophil function in infection and inflammation. The role of PtdIns(3,4,5)P3 in infection and inflammation, including in neutrophil biology, has been thoroughly reviewed elsewhere [18–26].

### Regulation of PtdIns(3,4,5)P3 signaling by lipid phosphatases

PtdIns(3,4,5)P3 signaling is activated in neutrophils by extracellular ligands such as chemokines or growth factors, while PtdIns(3,4,5)P3 synthesis by PI3K is balanced by the action of lipid phosphatases. The tumor suppressor protein phosphatase and tensin homologue (PTEN), a phosphatidylinositol 3'-phosphatase, converts PtdIns(3,4,5)P3 to PtdIns(4,5)P2, antagonizing the effect of PI3K and inhibiting PtdIns(3,4,5)P3 signaling [27,28]. Similarly, SHIP [27,29] and 5ptase IV [30,31] convert PtdIns(3,4,5)P3 to PtdIns(3,4)P2, thereby also exerting an inhibitory effect (Fig 1C).

#### PTEN

PTEN is a mammalian phosphatidylinositol 3'-phosphatase known to modulate a range of cellular functions in various cell types,

including neutrophils. PtdIns(3,4,5)P3 accumulates at the leading edge of chemotaxing cells [32–36]. Disruption of PTEN in the amoeba *Dictyostelium discoideum*, a chemotaxis model, results in enhanced and prolonged PtdIns(3,4,5)P3 production, actin polymerization, persistent multiple (or broad) pseudopodia, and impaired directional migration in response to a chemoattractant gradient [33,37]. PTEN's role in hematopoietic cells has been well investigated. Li *et al* [34] demonstrated that PTEN's intracellular localization and activity can be regulated by chemoattractants via the Rho family of GTPases. In their experiments, PTEN and RhoA co-localized at the back of stimulated neutrophils, and active RhoA increased PTEN phosphatase activity via its downstream effector, Rock, which directly phosphorylated PTEN with Cdc42 acting as an enhancer. PTEN phosphorylation is required for its stability and activity [34,38]. Papakonstanti *et al* [39] proposed a pathway in which RhoA is activated via p110 $\delta$  (see Fig 1B). Inhibition of RhoA resulted in misorientation of PTEN toward the front of the cell where Cdc42 localizes, and knockdown of PTEN in Jurkat T cells impaired chemotaxis in transwell migration assays. It appears that the RhoA activity at the back of polarizing cells is also regulated by PDZrhoGEF, which contains a Galpha12/13 coupled GRS domain and can be directly activated by G12/13. PDZrhoGEF is localized at the back of chemotaxing cells. Cells depleted of this protein exhibit multiple fronts and long tails [40,41]. It is noteworthy that the COOH terminus of PTEN contains a typical PSD-95/Dlg/ZO-1 (PDZ) binding motif. Indeed, PTEN has been reported to associate with several PDZ domain-containing proteins [42–47]. It is intriguing to speculate whether PTEN-PDZ domain interaction may play a role in regulating the biological function of PTEN in neutrophil chemotaxis.

Together, the published results allow us to propose a mechanism for PTEN's function in neutrophils: Localization of PTEN at the uropod locally inhibits PtdIns(3,4,5)P3 production at the back of the cell, thereby localizing PtdIns(3,4,5)P3 production and actin polymerization to the cell's leading edge and mediating directional sensing. Disruption of PTEN would therefore be expected to lead to uncontrolled propagation of PtdIns(3,4,5)P3 at the leading edge and consequent formation of multiple pseudopodia, frequent directional changes, and loss of directionality [48]. To investigate the exact role of PTEN in neutrophils, we generated a myeloid-specific PTEN knockout mouse. Stimulation of PTEN<sup>-/-</sup> mouse neutrophils with chemoattractant resulted in increased PtdIns(3,4,5)P3 synthesis, more exaggerated and sustained F-actin polymerization, elevated sensitivity to chemoattractant, and augmented ROS production [49]. Disruption of PTEN also led to enhanced phagocytosis, consistent with the essential role of phospholipids in phagocytosis [50–57]. Single-cell chemotaxis assays showed that PTEN<sup>-/-</sup> neutrophils had

#### Figure 1. Inositol phosphates and phosphoinositides.

(A) Inositol phosphates contain only inositol and phosphate and are therefore water soluble and cytoplasmic. Ins(1,4,5)P3 can be converted to Ins(1,3,4,5)P4 by InsP3K family members [143–145]. InsP7 is a higher inositol phosphate containing energetic pyrophosphate bonds [128,139] and is formed by InsP6K family members [130,136,177]. Phosphoinositides also contain inositol and phosphates, but in addition they contain two hydrophobic fatty acids and are therefore not water soluble and are mainly localized to membrane structures. (B) PI3K isoforms. Class I PI3Ks are heterodimeric proteins with a catalytic subunit and a regulatory subunit. Class IA PI3Ks consist of a catalytic subunit, p110 ( $\alpha$ ,  $\beta$ , or  $\delta$ ), and an adaptor subunit, p85 ( $\alpha$  or  $\beta$ ), and they are regulated by receptor tyrosine kinases. The only class IB member is p110 $\gamma$ , which is associated with p101 and p84/p87<sup>PIKAP</sup> regulatory subunits and is regulated by GPCRs such as chemokine receptors, C5a, and LTB4 [6,9]. (C) PtdIns(3,4,5)P3 signaling pathway. PtdIns(3,4,5)P3 exerts its function by mediating protein translocation via binding to their pleckstrin homolog (PH) domains. PH domain is a divergent protein module of approximately 120 amino acids found in many proteins involved in signal transduction [8,140,178–181]. There are more than 200 PH domains identified in the human genome. PH domains often mediate protein–protein or protein–phosphoinositide interactions. A subset of PH domains, including those in Btk, protein kinase B (PKB)/Akt, PLC- $\gamma$ , Gab1, PDK1, and Grp1, drive membrane translocation of their host proteins through specific, high-affinity recognition of PtdIns(3,4,5)P3. Only the PH domain-containing protein molecules on the plasma membrane can be activated, which in turn elicit various signal transduction pathways mediating neutrophil functions.

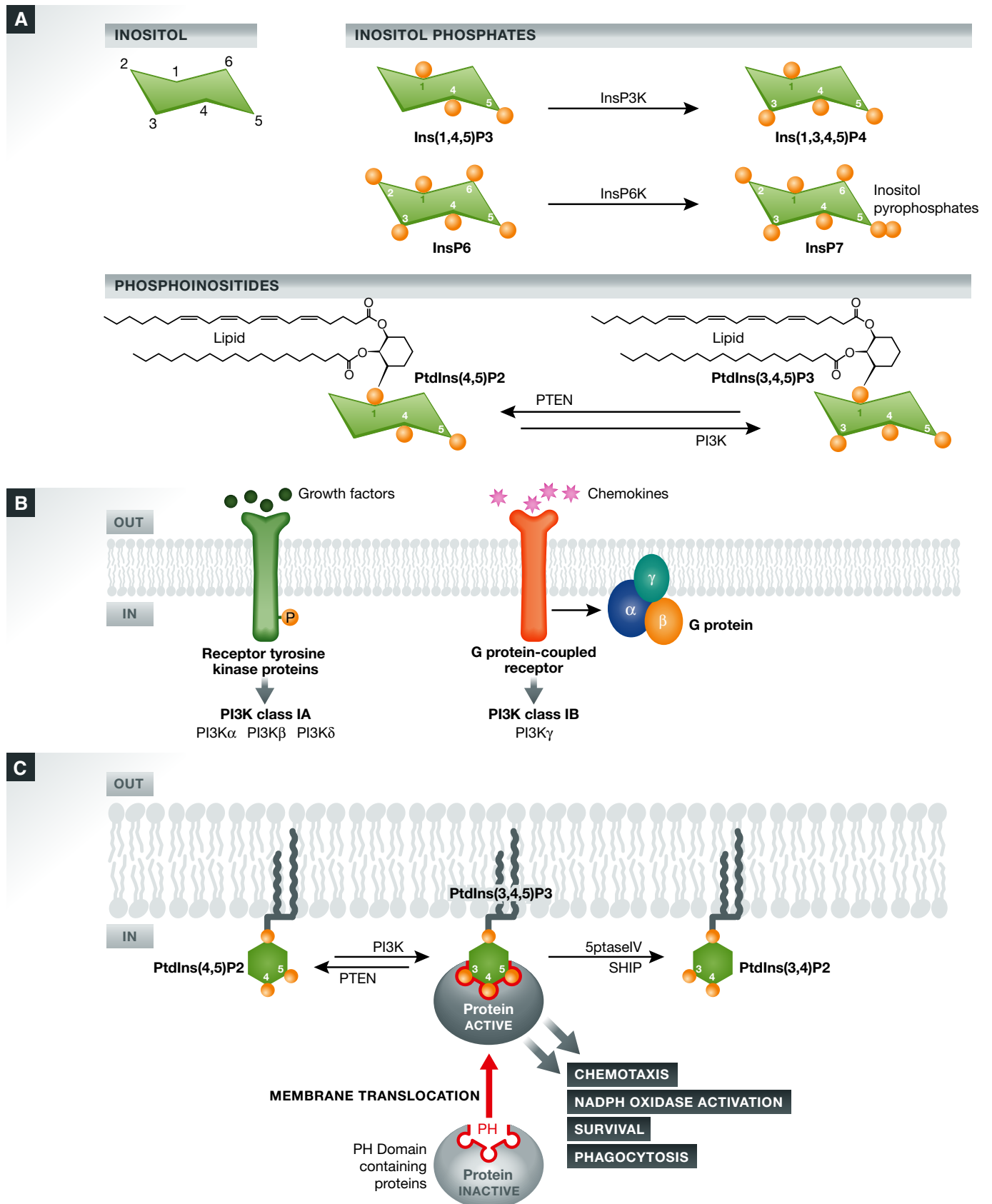


Figure 1.

small but significant defects in directionality, consistent with the mechanistic hypothesis that PTEN plays a role in mediating polarized PtdIns(3,4,5)P3 production at the leading edge of chemotaxing cells [34]. However, these neutrophils also moved quickly, and, as a result, overall chemotaxis (which is a function of both speed and directionality) was not affected. Similar results have also been observed in PTEN-deficient T cells [58,59]. PTEN's function in directional sensing in *Dictyostelium* and mammalian cells, including neutrophils, is therefore different. Of note, neutrophils isolated from mice carrying a "knock-in" allele of PI3K $\gamma$  showed GPCR-uncoupled PtdIns(3,4,5)P3 accumulation and displayed much greater impairment in directional cell migration in response to chemoattractants. Stimulated mutant macrophages did not polarize PtdIns(3,4,5)P3, and chemoattractant-elicited Rac activation was shortened due to enhanced PI3K-dependent activation of RacGAPs [60].

Consistent with the observed increased responsiveness of PTEN<sup>-/-</sup> neutrophils *in vitro*, recruitment of PTEN<sup>-/-</sup> neutrophils to inflamed mouse peritoneal cavities was significantly enhanced *in vivo* [49]. Using intravital video microscopy, we also observed enhanced neutrophil emigration from cremasteric muscle venules in PTEN knockout mice. PtdIns(3,4,5)P3 is a major downstream target of integrins and chemokine receptors and has been implicated in multiple leukocyte trafficking steps [20,61–76]. Unexpectedly, leukocyte adhesion was not increased when PtdIns(3,4,5)P3 signaling was enhanced via PTEN depletion, suggesting that PtdIns(3,4,5)P3 signaling is essential for stimuli-elicited cell adhesion but is not a limiting step. Enhanced neutrophil recruitment to the cremaster muscle in response to different stimuli is most likely due to faster neutrophil movement in the vascular bed, across the vascular endothelium, and into the muscle tissue. Neutrophil rolling influx, rolling speed, and the number of firmly adherent neutrophils were not altered in PTEN knockout mice. PTEN therefore acts as a negative regulator of neutrophil trafficking, and enhanced neutrophil recruitment in PTEN knockout mice is mainly caused by augmented trans-endothelial migration [77].

Recently, Heit *et al* [78] reported that PTEN is required for prioritizing and integrating responses to multiple chemotactic cues in certain situations. Thus, PTEN's exact function in neutrophils might rely on the number and type of chemoattractants, as well as the relative doses or route used to induce neutrophil inflammatory reactions. In the setting of multiple chemoattractants, neutrophils favor end-target chemoattractants such as fMLP and C5a over intermediary endogenous chemoattractants such as IL-8 and LTB4. Heit *et al* proposed a hierarchical model in which neutrophil migration toward end-target chemoattractants is mediated by p38 MAPK, whereas migration toward intermediary chemoattractants is mediated by PtdIns(3,4,5)P3. When faced with competing gradients of end-target and intermediary chemoattractants, PtdIns(3,4,5)P3 signaling was significantly reduced by p38 MAPK [79], a cross talk that might be mediated by PTEN. When neutrophils were exposed to an IL8 gradient, PTEN localized to the uropod of chemotaxing cells, thereby contributing to accumulation of PtdIns(3,4,5)P3 at the leading edge. In the presence of opposing fMLP gradients, PTEN was distributed throughout the entire cell circumference, thus inhibiting all PI3K activity and permitting "preferential" migration toward fMLP via phospholipase A2 and p38. As a consequence, PTEN<sup>-/-</sup> neutrophils cannot prioritize chemoattractants and are "distracted" by chemokines when moving toward bacterial chemoattractants

[78]. Another recent study showed that the p38 MAPK p38 $\delta$  and PKD1 regulate PTEN activity in neutrophils, thereby controlling their extravasation and chemotaxis during acute lung inflammation [80].

PTEN is also a key regulator of neutrophil death. Neutrophils normally have a very short life span and readily undergo spontaneous programmed cell death, which plays a crucial role in neutrophil homeostasis and the resolution of inflammation [81]. PtdIns(3,4,5)P3 signaling dramatically decreases during neutrophil death, and deactivation of PtdIns(3,4,5)P3 signaling has been identified as a critical event in neutrophil spontaneous death. PTEN-depleted neutrophils live much longer than wild-type neutrophils [82], and SHIP1-null neutrophils, in which the PtdIns(3,4,5)P3 pathway is upregulated, have an extended life span [83]. Conversely, activation of SHIP1 by NADPH oxidase-stimulated Lyn leads to enhanced neutrophil apoptosis [84]. These results are consistent with reports that neutrophil apoptosis is enhanced in PI3K $\gamma$ -deficient mice with reduced Akt activity [85,86], where Akt is a major survival factor downstream of PtdIns(3,4,5)P3 [87]. PtdIns(3,4,5)P3 production in apoptotic neutrophils is maintained by chemokines in an autocrine manner, which activate PI3K $\gamma$  via GPCRs. PI3K $\gamma$ , but not class IA PI3Ks, is negatively regulated by gradually accumulating ROS in apoptotic neutrophils, which suppress PI3K $\gamma$  activity by inhibiting an actin-mediated positive feedback loop [88]. However, a recent study has shown that granulocyte colony-stimulating factor (G-CSF) activation of Akt is insufficient to prolong neutrophil survival, and neutrophils treated with G-CSF undergo apoptosis even in the presence of high levels of activated Akt. Moreover, inhibition of Akt fails to alter neutrophil survival, suggesting that there are other pathways and factors mediating PtdIns(3,4,5)P3-elicited survival signaling [89].

Of note, some of PTEN's functions may not be mediated by degradation of PtdIns(3,4,5)P3. For instance, Anderson *et al* recently reported that superoxide production elicited by serum-opsonized bioparticles is mainly mediated by complement and CD18. This process requires class III PI3K and its product, PtdIns(3)P, and is independent of class I PI3K and its product, PtdIns(3,4,5)P3 [74,90]. Here, PTEN acts as a lipid phosphatase, removing the D3-positioned phosphate from PtdIns(3,4,5)P3, PtdIns(3,4)P2, and PtdIns(3)P. Therefore, its effect on phagocytosis-associated superoxide production is most likely mediated by its lipid phosphatase activity on PtdIns(3)P.

#### SHIP

SHIP (or SHIP1) is a 145-kD SH2-containing inositol-5-phosphatase that is widely expressed in hematopoietic cells [27,29,91,92]. It was first identified as a tyrosine phosphoprotein associated with Shc in response to numerous cytokines. Hematopoietic cell-specific SHIP1 is responsible for the majority of phosphatidylinositol 5'-phosphatase activity in neutrophils, while the ubiquitously expressed SHIP2 only plays a minor role in hematopoietic cells. SHIP1 blunts PI3K-initiated signaling and is known to negatively regulate various cellular processes, such as phagocytosis, cell migration, degranulation, cell survival, proliferation, differentiation, and sensitivity to chemokines [93–96]. SHIP1-deficient mice exhibit chronic progressive hyperplasia of myeloid cells, perhaps at the expense of B-cell production. SHIP1 knockout myeloid progenitors show enhanced responsiveness to cytokines and are less susceptible to apoptotic

stimuli *in vitro*, and while increased proliferation and decreased apoptosis may contribute to the expanded hematopoietic stem cell (HSC) compartment in SHIP1 knockout mice, these HSCs are also compromised in their ability to home and repopulate [83,92]. SHIP1 expression was initially thought to be restricted to hematopoietic cells, but more recently it has become apparent that most bone marrow (BM) and blood cells express SHIP1, and Hazen *et al* [97] demonstrated SHIP1 expression and functional activity in non-hematopoietic BM cells. Furthermore, SHIP1 deficiency enables long-term reconstitution of the hematopoietic inductive BM micro-environment [98].

Although excessive PtdIns(3,4,5)P3 production in PTEN<sup>-/-</sup> neutrophils does not cause significant chemotaxis defects, one report suggests that SHIP1 knockout results in chemotaxis defects [99]. Findings from our own lab show that SHIP1 plays a predominant role in cell adhesion, rather than chemoattractant sensing, during cell migration. SHIP1<sup>-/-</sup> neutrophils were much more adherent than wild-type cells by engaging integrins. In suspension, PtdIns(3,4,5)P3 production in SHIP1<sup>-/-</sup> neutrophils remained constant when stimulated with chemoattractant, in contrast to adherent SHIP1<sup>-/-</sup> neutrophils, which showed marked upregulation of PtdIns(3,4,5)P3 when stimulated. As a consequence, actin polymerization was restricted to the leading edge of suspended SHIP1<sup>-/-</sup> neutrophils, with cell polarity lost upon adhesion [100]. During migration, SHIP1 appears to act as a negative regulator of PtdIns(3,4,5)P3 formation at the cell–substratum interface, preventing the formation of top-down PtdIns(3,4,5)P3 polarity and facilitating normal cell attachment and detachment during chemotaxis (Fig 2A).

In the same study, we also examined neutrophil migration *in vivo*. Surprisingly, despite the chemotaxis defect, recruitment of SHIP1<sup>-/-</sup> neutrophils to the inflamed peritoneal cavity was significantly enhanced. We hypothesize that neutrophils passing through the endothelium and entering the tissue control their migration using two independent directional cues: a lateral front-to-back polarity mediated by chemotactic signaling and a dorsal–ventral polarity controlled by adhesion-mediated signaling. SHIP1 activity is regulated by adhesion and plays a critical role in balancing these two types of polarity in neutrophils. Loss of SHIP1 leads to upregulation of dorsal–ventral polarity, preventing neutrophils from effective lateral migration on surfaces. However, this dorsal–ventral polarity promotes top-down migration of neutrophils through soft surfaces such as the gaps between endothelial cells [100] (Fig 2B).

Although SHIP1 is thought to be enzymatically active in the cytosol, its activity is ultimately determined by its membrane localization [101]. Recruitment of SHIP1 to the plasma membrane is regulated by its association with adapter proteins (e.g. Shc, Grb2, Dok3), scaffolding proteins (e.g. Gab1/2), and by direct association with tyrosine-phosphorylated receptors via its SH2 domain. These interactions require tyrosine phosphorylation of SHIP1 at the NPXY motif [102–105]. We have observed that cell adhesion, but not chemoattractant stimulation, leads to tyrosine phosphorylation of SHIP1. In addition, SHIP1 can interact with FAK and Lyn upon cell adhesion and  $\beta_3$  integrin in both suspension and upon cell adhesion. This indicates that adhesion results in the recruitment of SHIP1 to the membrane where it can act on the PtdIns(3,4,5)P3 produced during cell adhesion [100]. It has previously been shown that Lyn, a Src family tyrosine kinase, regulates SHIP1 phosphorylation in integrin  $\alpha_{IIb}\beta_3$ -mediated adhesion and signaling in platelets [106].

Unlike PTEN-deficient neutrophils, in which ROS production is elevated, loss of SHIP1 leads to reduced ROS production in neutrophils in suspension upon stimulation with chemoattractant. In neutrophils, ROS are mainly produced by the phagocyte NADPH oxidase (also known as the NOX2 complex) [107–111]. During cell activation, the cytosolic components of the enzyme, namely p47phox, p67phox, Rac2, and p40phox, are recruited to the membrane to form a complex with its membrane components, p22phox and gp91 complex. Assembly of the NADPH oxidase complex catalyzes the conversion of molecular oxygen to superoxide, which is known to facilitate the destruction of invading pathogens [81,88,112–114]. Although PtdIns(3,4,5)P3 and PI3Ks have been identified as key regulators of NADPH oxidase activation, recruitment of cytosolic p47phox and p40phox to the NADPH oxidase complex requires the presence of the class III PI3K product, PtdIns(3)P, and the product of SHIP1, PtdIns(3,4)P2 [73,74,87,115–123]. Reduced ROS production in SHIP1<sup>-/-</sup> neutrophils is primarily due to decreased PtdIns(3,4)P2 levels, which correlates with reduced recruitment of p40phox and p47phox to the NADPH oxidase complex [100]. However, when SHIP1<sup>-/-</sup> neutrophils are primed and permitted to adhere to fibronectin, they produce very high levels of ROS. This is most likely due to the involvement of SHIP1<sup>-/-</sup> in integrin-mediated PtdIns(3,4,5)P3 signaling, with increased PtdIns(3,4,5)P3 signaling overriding the effect of decreased PtdIns(3,4)P2 levels [100].

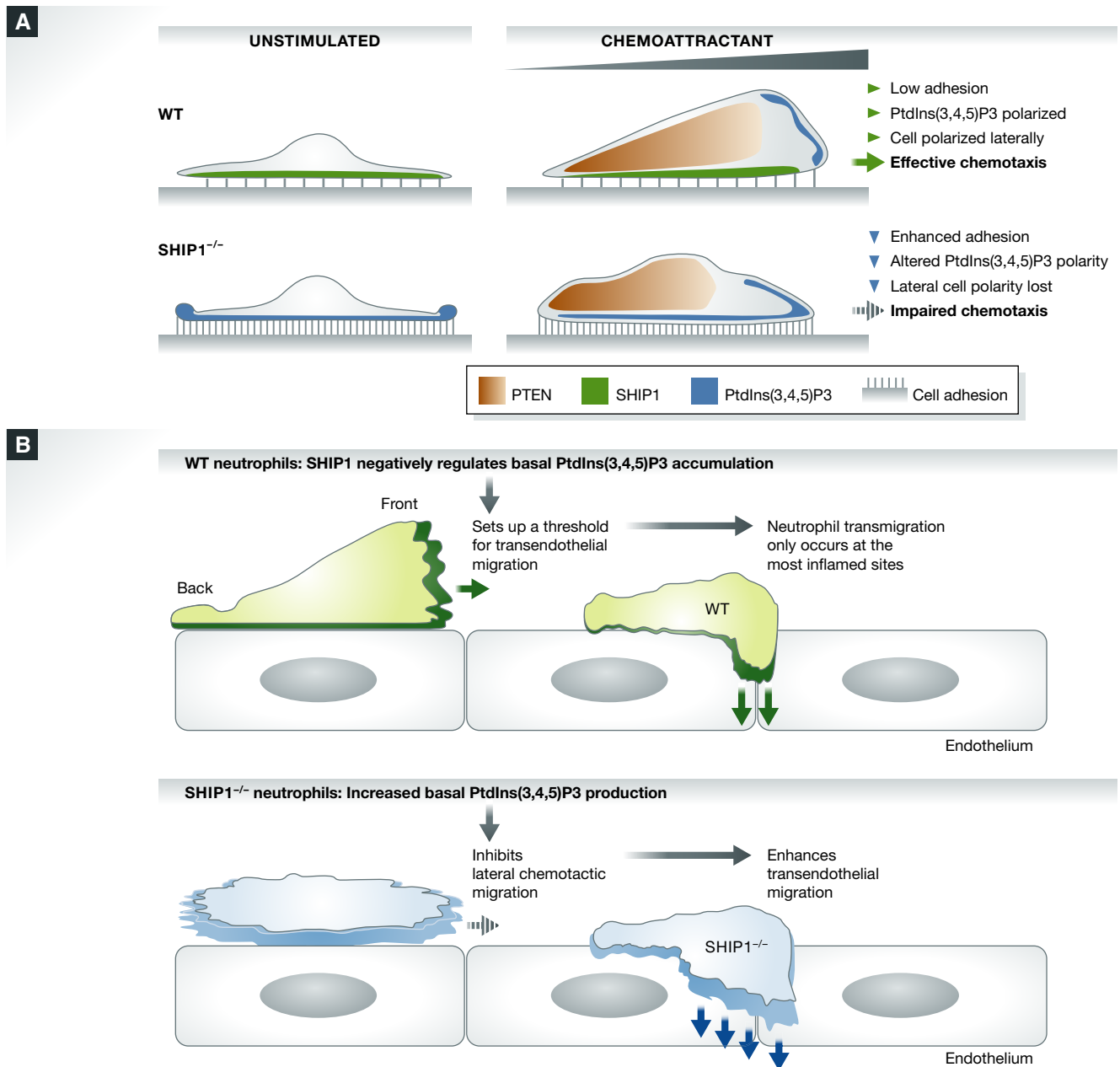
The PtdIns(3,4,5)P3 signaling pathway can be negatively regulated by lipid phosphatases SHIP1 and PTEN in neutrophils. The activity and subcellular localization of these lipid phosphatases are tightly controlled. How temporal and spatial regulation contributes to various neutrophil functions remains ill-defined and needs to be further investigated.

## Regulation of PtdIns(3,4,5)P3 signaling by inositol phosphates

PtdIns(3,4,5)P3 signaling was previously thought to be solely dependent on the concentration of PtdIns(3,4,5)P3 in the cell membrane [6]. We discovered that two inositol phosphates, InsP7 and Ins(1,3,4,5)P4, compete with PtdIns(3,4,5)P3 for PH domain binding and suppress PH domain translocation, providing a novel mode of regulation for PtdIns(3,4,5)P3 signaling in neutrophils [124,125] (Fig 3).

### InsP7

InsP7 has been implicated in a variety of cellular functions such as vesicular trafficking, apoptosis, endocytosis, DNA repair/recombination, and maintenance of telomere length [126]. InsP7 arises from pyrophosphorylation of InsP6, the most abundant inositol phosphate in mammalian cells [127]. The enzymes that catalyze the synthesis of InsP7 comprise a family of InsP6 kinases (InsP6K) including InsP6K1, InsP6K2, and InsP6K3 [128,129] (Fig 1). InsP6K1 and InsP6K2 are highly expressed in neutrophils, while InsP6K3 is essentially undetectable. In neutrophils, InsP6K1 and InsP6K2 isoforms have non-redundant roles. InsP6K1 seems to be equally distributed in the nucleus and cytoplasm, while, in contrast, InsP6K2 appears to be almost exclusively nuclear in localization [130]. In a recent study, we reported that InsP6K1 could regulate

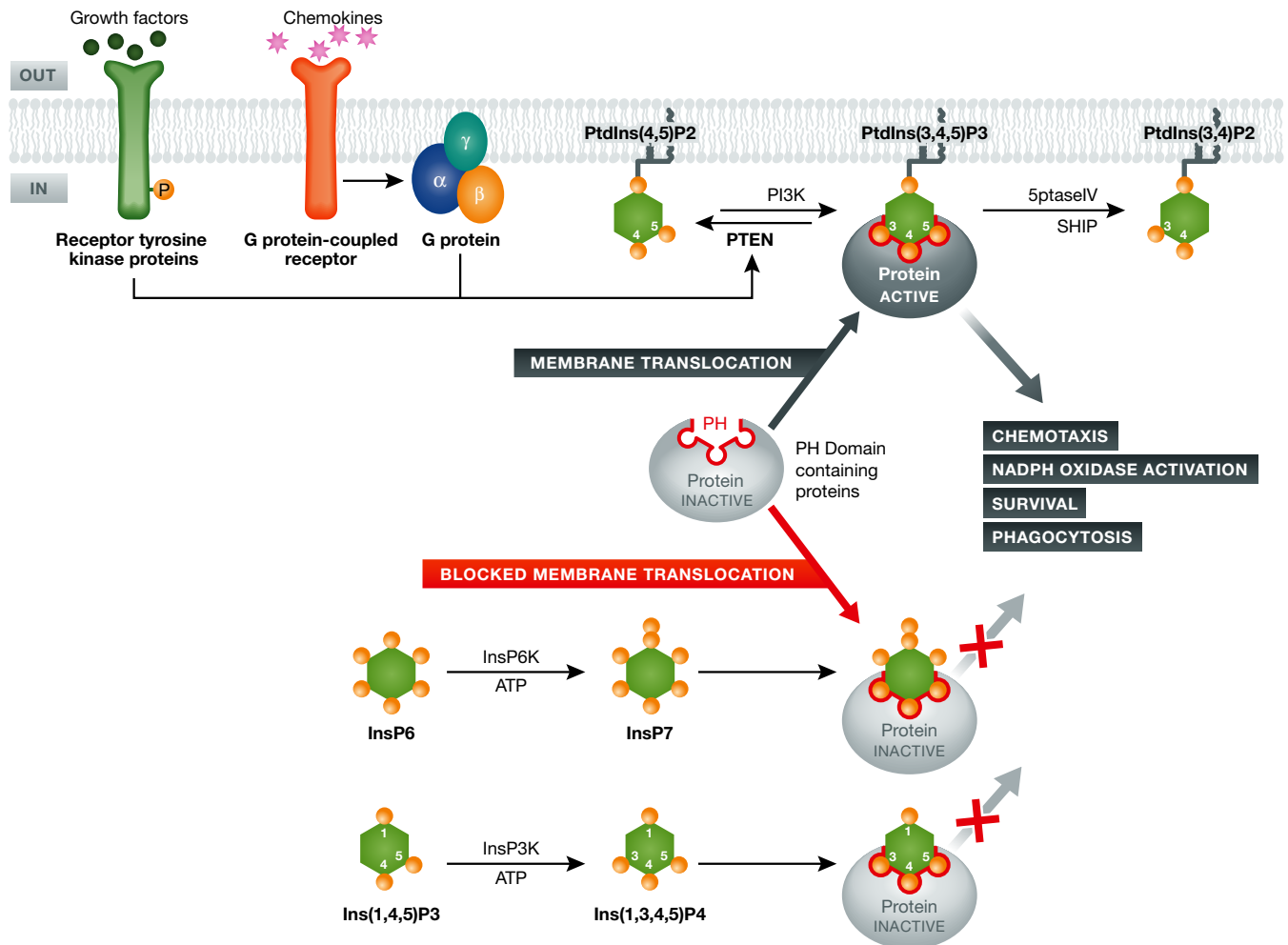


**Figure 2. The role of SHIP1 and PTEN in neutrophil chemotactic migration.**

(A) SHIP1 and PTEN act via different receptor-regulated processes to control spatial accumulation of PtdIns(3,4,5)P3 and establish a proper anterior–posterior PtdIns(3,4,5)P3 compass. SHIP1 acts as a negative regulator of integrin-mediated cell adhesion in neutrophils. In wild-type neutrophils, integrin-mediated cell adhesion results in PtdIns(3,4,5)P3 production at the sites of cell adhesion. Concurrently, SHIP1 at the cell–substratum interface is engaged, phosphorylated, and activated. This activity is crucial for dephosphorylating the PtdIns(3,4,5)P3 formed during cell adhesion. With the combined actions of both SHIP1 and PTEN, PtdIns(3,4,5)P3 polarity is maintained at the leading edge, neutrophils polarize, and there is effective cell migration. PTEN is localized to the rear end of a migrating cell to facilitate the accumulation of PtdIns(3,4,5)P3 at the anterior end, and SHIP1 is active at the cell–substratum interface to abolish the PtdIns(3,4,5)P3 gradient being formed by integrin activation. With loss of SHIP1, adhesion-mediated PtdIns(3,4,5)P3 formation is uncontrolled, resulting in the formation of “top-down” PtdIns(3,4,5)P3 polarity. Increased PtdIns(3,4,5)P3 levels enhance cell adhesion. This leads to activation of various PtdIns(3,4,5)P3 effector proteins and consequently results in impaired chemotaxis. (B) SHIP1 plays a critical role in neutrophil transendothelial migration by balancing the lateral and dorsal–ventral polarity of transmigrating neutrophils.

PtdIns(3,4,5)P3 signaling in neutrophils [131]; disruption of InsP6K1 enhanced PtdIns(3,4,5)P3 signaling, and, as a consequence, these neutrophils exhibited elevated phagocytic and bactericidal capabilities and amplified NADPH oxidase-mediated superoxide

production. These findings established a novel role for InsP7 in the regulation of cellular signal transduction pathways in neutrophils and provided a novel mechanism for modulating PtdIns(3,4,5)P3 signaling in mammalian cells [131].



**Figure 3. Regulation of PtdIns(3,4,5)P3 signaling by inositol phosphates in neutrophils.**

InsP7 or Ins(1,3,4,5)P4 can compete with PtdIns(3,4,5)P3 for PH domain binding and thus prevent their recruitment onto the plasma membrane and consequently inhibit their activation.

Consistent with its regulatory role in mediating chemoattractant-elicited signals, InsP7 levels are tightly regulated in neutrophils. Unstimulated neutrophils contain a substantial amount of InsP7, with levels decreasingly markedly and rapidly upon stimulation with chemoattractants [131]. Cigarette smoke extract (CSE) and nicotine also reduce InsP7 levels in aging neutrophils, which subsequently leads to suppression of Akt deactivation and delayed neutrophil death. Delayed neutrophil death contributes to the pathogenesis of CS-induced COPD. Disruption of InsP6K1 consistently augments CS-induced neutrophil accumulation and lung damage [132]. Recently, Chakraborty *et al* [133] showed that InsP7 also negatively regulates PtdIns(3,4,5)P3/Akt signaling in glucose homeostasis and protein translation. InsP7 inhibits Akt by acting at the PH domain of Akt to prevent its plasma membrane translocation, phosphorylation, and activation by PDK1 [133]. Intriguingly, in contrast to our observation that chemoattractant inhibits InsP7 formation, Chakraborty *et al* showed that growth factors stimulate InsP7 production. Therefore, the inhibition of Akt signaling by InsP7 may be a general signal transduction phenomenon. However, its regulatory mechanism and resulting physiologic consequences might be cell or system specific.

The mechanisms by which InsP7 production is suppressed in chemoattractant- and CSE-stimulated neutrophils are still largely unknown and need to be investigated further. However, these mechanisms are likely to involve activation of inositol pyrophosphate phosphatase and/or deactivation of InsP6 kinase.

Although InsP6K1 disruption augments PtdIns(3,4,5)P3 signaling, it fails to augment cell adhesion, sensitivity, or migration speed in neutrophils [131]. These results are somewhat different to the migratory phenotypes observed in PTEN-null neutrophils [49]. Although overall chemotactic migration is relatively normal, PTEN disruption in neutrophils results in mildly impaired directionality, enhanced sensitivity to chemoattractant stimulation, and slightly increased migration speed [49]. These distinct effects are likely to occur as a result of different temporal and spatial regulation of PTEN and InsP6K1 in neutrophils. PTEN activity is increased, and its subcellular localization is altered, after chemoattractant stimulation [34]. In contrast, InsP6K1 levels are high in unstimulated neutrophils but are significantly reduced after chemoattractant stimulation. The mechanisms by which PTEN and InsP6K1 regulate PtdIns(3,4,5)P3 signaling are also different; PTEN regulates PtdIns

(3,4,5)P3 levels and controls neutrophil function via a number of downstream pathways, while InsP6K1 deletion is not associated with changes in PtdIns(3,4,5)P3 or its downstream signaling cascades, but instead is limited to its action on Akt. In addition, PTEN appears to act as a housekeeping gene, while InsP6K1 exhibits tissue-specific expression, and there is functional redundancy with InsP6K2 and/or InsP6K3 in some cell types [130,134–136]. These results highlight the complexity of the PtdIns(3,4,5)P3 signaling network and suggest that different temporal and spatial regulation of this signaling molecule can lead to distinct cellular effects.

#### Ins(1,3,4,5)P4

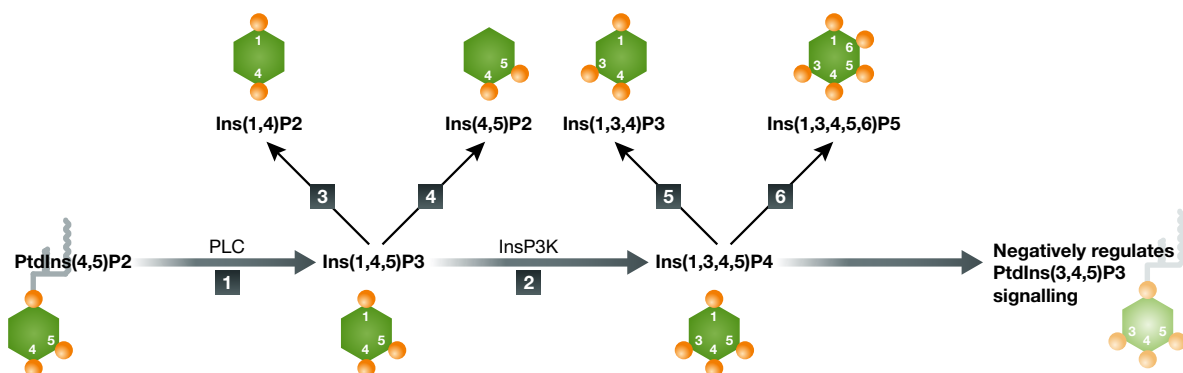
Ins(1,3,4,5)P4 is the predominant InsP4 isoform in neutrophils [137,138], intracellular levels of which are regulated by inositol phosphate kinases and phosphatases [128,139] (Fig 4). The major metabolic pathway generating Ins(1,3,4,5)P4 is via Ins(1,4,5)P3, with Ins(1,4,5)P3 formed from the hydrolysis of PtdIns(4,5)P2 by PLC [116,140–142]. Ins(1,3,4,5)P4 is eventually formed by InsP3Ks, which belong to a highly conserved family of inositol phosphate kinases [128,136,139]. Members of this family include InsP6Ks, InsP3Ks, and IPMK [143–145]. There are three InsP3K isoforms in mammalian cells, designated A, B, and C [143–145]. The gene encoding InsP3KA is exclusively expressed in specific subpopulations of neurons in the central nervous system and in the testis [143], while isoforms B and C are ubiquitously expressed [143–145].

The physiologic functions of InsP3K in hematopoietic cells were recently studied using InsP3K knockout mice [146,147]. The InsP3KB isoform appeared to contribute the majority of InsP3K activity in T and B cells [148–151]. Disruption of InsP3KB led to a dramatic decrease in cellular Ins(1,3,4,5)P4 levels, impaired T-cell development, and defective thymocyte selection. Huang *et al* demonstrated that Ins(1,3,4,5)P4 can bind to the PH domain of the Tec family tyrosine kinase Itk, which plays an important role in TCR signaling. Interestingly, this binding altered the conformation of the Itk PH domain and subsequently promoted, rather than suppressed, PH domain binding to PtdIns(3,4,5)P3 in T cells [152]. Disruption of InsP3KB induced B-cell death and impaired B-cell development [153,154]. Miller *et al* [153] reported that Ins(1,3,4,5)P4 acts by modulating store-operated calcium channels, with elevated calcium

influx detected in InsP3KB null B cells. InsP3K has been reported to be a potential modulator of calcium mobilization, since it can decrease the level of Ins(1,4,5)P3, which mediates calcium release from internal stores by converting it to Ins(1,3,4,5)P4. Surprisingly, no substantial defects in Ins(1,4,5)P3 quantity or calcium mobilization were detected in InsP3KB-null T cells [148,149].

InsP3KB is also the major InsP3K isoform in neutrophils. Disruption of the InsP3KB gene leads to alteration of myelopoiesis, hyperactivation of neutrophils, and dysregulation of innate immunity [125,155,156]. Ins(1,3,4,5)P4 depletion in InsP3KB-deficient neutrophils enhances membrane translocation of the PtdIns(3,4,5)P3-specific PH domain, thus augmenting PtdIns(3,4,5)P3-mediated downstream signaling. This leads to enhanced sensitivity to chemoattractants, elevated superoxide production, and enhanced neutrophil recruitment to inflamed peritoneal cavities in mice [125]. Although loss of InsP3KB leads to elevated neutrophil recruitment and function, bacterial killing was actually reduced *in vivo*. This was mainly due to B- and T-cell defects, which led to significantly reduced levels of immunoglobulin and impaired opsonization and phagocytosis [125]. Disruption of InsP3KB does not affect overall calcium signaling in the presence of extracellular calcium in neutrophils [125]. However, a more detailed investigation revealed significantly decreased calcium release from intracellular stores and enhanced calcium influx through store-operated calcium channels in InsP3KB-null neutrophils stimulated with chemokines. Reduced calcium release from intracellular stores appears to be a result of calcium depletion from the store (Y. Jia and H.R. Luo, unpublished data).

In neutrophils, chemoattractant stimulation triggers a potent and rapid elevation in Ins(1,3,4,5)P4, indicating that Ins(1,3,4,5)P4 levels are regulated by receptor occupancy. InsP3K is not constitutively active in neutrophils, and its activity in unstimulated neutrophils is relatively low. Activity is, however, dramatically increased by chemoattractant stimulation. The low basal InsP3K activity might be necessary for chemoattractant-stimulated neutrophils to generate or maintain an initially high concentration of Ins(1,4,5)P3, which is essential for downstream calcium signaling. Alternatively, the regulation of InsP3K by chemoattractants may simply be another mechanism to finely tune intracellular Ins(1,3,4,5)P4 concentrations.



**Figure 4. The metabolic pathways controlling intracellular Ins(1,3,4,5)P4 levels.**

1. Phospholipase C (PLC). 2. Ins(1,4,5)P3 kinase (InsP3K). 3. Ins(1,4,5)P3 5-phosphatase. 4. Ins(1,4,5)P3 1-phosphatase. 5. Inositol polyphosphate 5-phosphatase. 6. Ins(1,3,4,5)P4 6-kinase. All these enzymes are ubiquitously expressed.



The molecular mechanism underlying the chemoattractant-induced enhancement of InsP3K activity is still largely unknown. Recent studies have shown that all three InsP3K isoforms contain an F-actin binding domain, and InsP3KB is highly localized to the leading edge of polarized neutrophils [144,157–159]. Whether the actin–InsP3KB interaction plays any role in modulating InsP3KB activity needs to be determined. In addition, since all three isoforms contain a calmodulin binding motif, the InsP3KB activity in neutrophils may also be regulated by calcium [125].

### Targeting PtdIns(3,4,5)P3 signal pathway in infectious and inflammatory diseases

Excessive neutrophil accumulation or hyper-responsiveness of neutrophils can be detrimental to the host system, and the response of neutrophils to inflammatory stimuli needs to be tightly controlled. Neutrophils contribute to the pathogenesis of a number of autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and systemic lupus erythematosus (SLE). Since receptor-mediated signal transduction in neutrophils relies heavily on PI3K isoforms, small molecule inhibitors to class I PI3Ks should counteract the undesirable pro-inflammatory effects of neutrophils in inflammatory conditions. Over the last few years, a number of isoform-specific PI3K inhibitors have been developed, and some of them are already being tested in clinical trials for various inflammatory diseases [68,75,160–167]. Since SHIP1 inhibition leads to massive myeloid infiltration of the lungs and progressive inflammation, pharmacological activation of SHIP1 has emerged as a therapeutic strategy for inflammatory pulmonary diseases. A small molecule SHIP1 activator AQX-1125, which binds to the C2 domain of SHIP1 to increase its catalytic activity, is currently in Phase II clinical trials. Since SHIP1 expression is restricted to hematopoietic cells, targeting SHIP1 is expected to limit unwanted off-target side effects. AQX-1125 suppresses Akt phosphorylation, reduces cytokine production, and inhibits neutrophil activation and chemotaxis [168,169].

There are also cases in which enhanced neutrophil recruitment and function would be expected to be clinically beneficial. For example, neutropenia and related infections are the most important dose-limiting toxicities of cytotoxic anti-cancer chemo- and radiotherapies, which impact on both the quality and quantity of life. One obvious strategy for treating neutropenia-related infections is to administer broad-spectrum antibiotics to neutropenic patients. However, not all patients respond to antibiotic treatments, and this method carries the risk of inducing antibiotic resistance. An alternative approach is G-CSF (filgrastim) treatment [170], which is now used clinically to restore neutrophil counts in neutropenia-related pneumonia patients via stimulation of the BM to produce more neutrophils. However, this therapy does not always work if the BM has not recovered from chemotherapy, and treatment is associated with side effects such as bone pain, headache, fatigue, and nausea. Long-term use of G-CSF might also increase the risk of leukemia [170–173]. Therefore, directly elevating innate immunity by promoting neutrophil recruitment and function is a reasonable alternative strategy under these circumstances. We have recently shown that neutrophil function can be enhanced in neutropenic conditions by activating intracellular PtdIns(3,4,5)P3 signaling [174]; significantly, more neutrophils were recruited to inflamed lungs during

neutropenia-associated pneumonia in myeloid-specific PTEN knock-out mice. Using an adoptive transfer technique, we demonstrated that enhanced neutrophil recruitment was caused directly by PTEN depletion in neutrophils. In addition, disruption of PTEN increased recruitment of macrophages and elevated levels of pro-inflammatory cytokines/chemokines in the inflamed lungs, which might also have contributed to enhanced neutrophil recruitment. Finally, we provided direct evidence that the enhanced neutrophil function caused by elevated PtdIns(3,4,5)P3 signaling can alleviate pneumonia-associated lung damage and decrease pneumonia-elicited mortality. Furthermore, we showed that activation of PtdIns(3,4,5)P3 signaling by PTEN disruption or using the SF1670 PTEN inhibitor increased the efficacy of granulocyte transfusion in neutropenia-related pneumonia [175]. It is noteworthy that although we consistently observed enhanced neutrophil migration in PTEN-deficient mice suffering from *E. coli* pneumonia, Schabbauer *et al* recently reported that the absence of myeloid cell-associated PTEN dampens pulmonary inflammation, reduces neutrophil influx, and augments the phagocytic properties of macrophages, which ultimately resulted in decreased tissue injury and improved survival during murine pneumococcal pneumonia. PTEN may differentially regulate the attraction of neutrophils depending on the inducing agent [176].

Elevating PtdIns(3,4,5)P3 can augment neutrophil recruitment and function in host defense responses. However, hyperactivation of neutrophils can lead to unwanted tissue damage and inflammation. In addition, some activators of PtdIns(3,4,5)P3 signaling may not be appropriate therapeutic targets for clinical intervention due to their carcinogenic or other harmful effects. This balance between benefit and harm remains a challenge in targeting PtdIns(3,4,5)P3 signaling in inflammatory conditions. The PtdIns(3,4,5)P3 signaling pathway is negatively regulated by lipid phosphatases and inositol phosphates, which consequently play a critical role in controlling neutrophil function and thus would be expected to act as ideal therapeutic targets for enhancing or suppressing innate immune responses.

#### Sidebar A: In need of answers

- (i) How are the activity and subcellular localization of PTEN and SHIP1 regulated in neutrophils? How do temporal and spatial regulation contribute to various neutrophil functions?
- (ii) What molecular mechanism underpins chemoattractant-induced enhancement of InsP3KB activity in neutrophils? Do actin–InsP3KB interactions play any role in modulating InsP3KB activity?
- (iii) The mechanisms by which InsP7 production is suppressed in chemoattractant- and CSE-stimulated neutrophils.
- (iv) When targeting PtdIns(3,4,5)P3 signaling in the clinical setting, how can a balance between the beneficial and detrimental effects be achieved?

#### Conflict of interest

The authors declare that they have no conflict of interest.

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