

Targeting phosphoinositide 3-kinase γ to fight inflammation and more

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Summary

The family of class I phosphoinositide-3-kinase (PI3K) is composed of four lipid kinases involved at multiple levels in innate and adaptive immune responses. Class I PI3Ks are divided into two subclasses, IA and IB, sharing a similar catalytic core. Whereas class IA PI3Ks are primarily activated by receptor tyrosine kinases, the unique element of class IB PI3K (PI3K γ) is activated by G protein coupled receptors (GPCRs), like chemokine receptors. PI3K γ is mainly expressed in leukocytes where it plays a significant role in chemotaxis. Here, we report recent ad-

vances in the analysis of the role of PI3K γ in leukocytes and in endothelial cells. Results, derived from studies based on both pharmacological and genetic approaches, confirm PI3K γ as an attractive target for drug discovery. PI3K γ specific inhibition has gained increasing attention for the treatment of allergic, autoimmune and inflammatory diseases. Development of inhibitors has already provided series of hits, whose efficacy is currently under scrutiny worldwide.

Keywords

Inflammation, leukocyte trafficking / recruitment, signal transduction, endothelial cells

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Introduction

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases initially co-purified with oncoproteins (1). Later on, this family was described to be involved in many homeostatic mechanisms that include cell growth, cell differentiation, metabolism and immune function.

Once activated by receptors located at the cell surface, PI3Ks phosphorylate the D-3 position of the inositol ring of phosphoinositides. In particular, PI3Ks of class I are all involved in the phosphorylation of phosphatidyl-inositol (4,5)-bisphosphate [PtdIns(4,5) P_2] and produce phosphatidyl-inositol (3,4,5)-trisphosphate [PtdIns(3,4,5) P_3]. This second messenger acts as a docking site at the plasma membrane, recruiting and activating proteins containing phospholipid-binding domains such as the pleckstrin homology (PH) motif (2, 3). Primarily, these downstream PI3K effectors include protein kinases that promote cell growth, survival and proliferation such as PKB/Akt, PDK1 and the Tec family kinases (Fig. 1). However, downstream PI3K effectors also consist of scaffolding proteins that mediate the assembly of key signaling complexes, such as Gab2 (4), and of GTPase activating proteins (GAPs), such as Tsc1, Tsc2 and Arh-

GAP15 (4, 5), as well as guanine nucleotide exchange factor (GEFs), such as P-Rex, Swap70 and Vav (6–9). These GAPs and GEFs, in turn, regulate the activity of GTPases of the Ras superfamily, eventually modulating cytoskeletal remodeling, membrane trafficking and cell motility (Fig. 1). Negative control of the PI3K pathway is ensured by protein phosphatases, like the phosphatase and tensin homolog on chromosome ten (PTEN) that reconverts PtdIns(3,4,5) P_3 into PtdIns(4,5) P_2 .

The diverse substrate specificity, together with the differences in the isoform structures, enable the subdivision of the PI3K family into three classes that mediate distinct functions correlated to their cell distribution. Among PI3K classes, class I is the best characterized, comprises four members and is further subdivided into two subclasses, named IA and IB, respectively. All class I PI3Ks consist of a regulatory and a catalytic subunit, sharing highest homology in the ATP- and substrate-binding site. The three members of subclass IA are named PI3K α , PI3K β and PI3K δ and are primarily activated by receptor tyrosine kinases. PI3K γ the unique member of class IB, is activated by G protein-coupled receptors (GPCRs) via binding of $\beta\gamma$ subunits of G proteins. This activation is facilitated by the regulatory subunits p101 and p84/87 that tether the enzyme to the plasma membrane (10).

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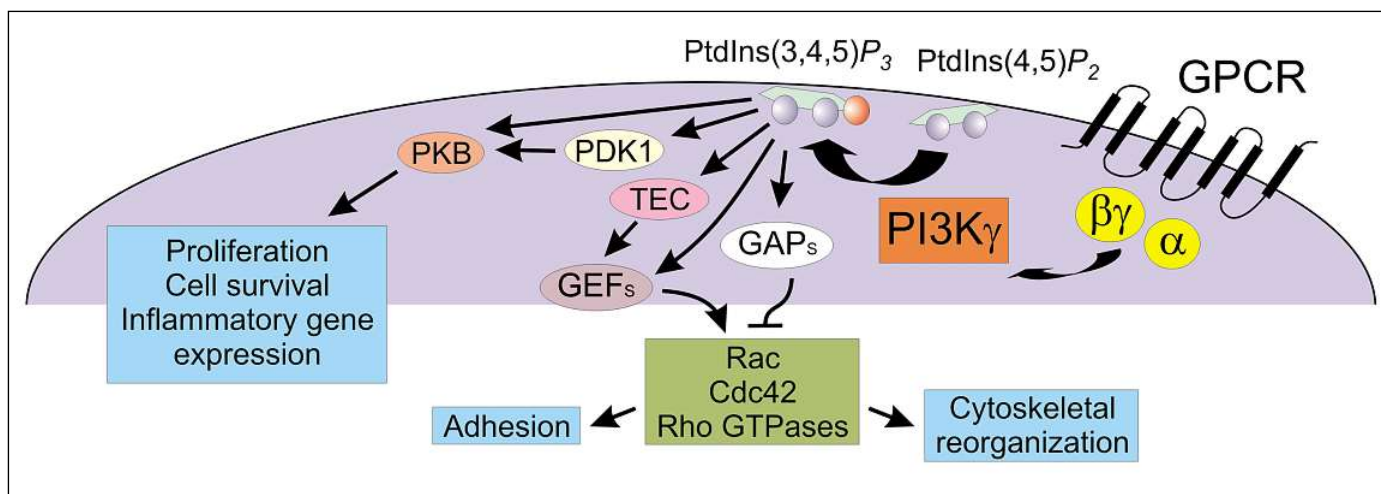


Figure 1: Schematic representation of the main signal transduction pathways triggered in leukocytes by PI3K γ activation and subsequent PtdIns(3,4,5)P $_3$ production.

The first pharmacological inhibitors described for class I PI3Ks are wortmannin and LY294002 (11). These compounds are unable to discriminate between different PI3K isoforms and also inhibit enzymes that are structurally similar to PI3Ks, like the target of rapamycin (mTOR) or myosin light-chain kinase (12). A second generation of isoform specific inhibitors are being generated and some have been already described (13). The use of these molecules, as well as gene targeting technology, has permitted to identify specific roles for each member of the PI3K family.

Class I PI3K α and β are ubiquitously expressed, and knock-out mice for their genes have proven to die at an embryonic stage, thus indicating a role for these isoforms in mammalian development (14, 15). On the other hand, PI3K γ and δ are expressed predominantly, although not exclusively, in leukocytes and gene targeting experiments indicated that these two enzymes are involved in both innate and adaptive immune responses (3, 10, 13, 16). In particular, PI3K γ functions as a chemokine sensor regulating leukocyte migration (5, 17–19). Nonetheless, PI3K γ is also expressed outside the hematopoietic system where it plays other distinct specific roles. For example, PI3K γ is present in cardiomyocytes, where it is involved in the regulation of contractility (20, 21). Furthermore, this enzyme can be found in vessels, in both smooth muscle (22) and endothelial cells (23), and appears involved in blood pressure, shear stress responses as well as angiogenesis (10).

In this review, we intend to focus on the specific role of PI3K γ in leukocytes and endothelial cells, addressing the functions that characterize this enzyme as an attractive target for anti-inflammatory drugs.

PI3K γ in leukocyte function

Mice lacking PI3K γ do not present any overt phenotype and appear viable and fertile. Nevertheless, they display abnormalities when their immune system is stressed. The lack of PI3K γ impairs the ability of neutrophils and macrophages to respond to several GPCR stimuli, such as chemokines, activated complement fragments and N-formyl-methionyl-leucylphenylalanine (fMLP)

(17–19, 24, 25). For example, PI3K γ -null neutrophils stimulated with fMLP display not only a chemotactic defect but also a marked reduction in their ability to trigger NADPH oxidase activity and produce reactive oxygen species (ROS) of the O $_2^-$ type (26). In normal conditions, the exposure of human neutrophils to proinflammatory cytokines induces a PI3K-dependent increase in ROS production that plays an important role in the induction of the damage to surrounding tissue. In this process, PI3K γ acts in concert to other PI3Ks, and the role of the specific PI3K isoforms in this process has just lately started to be unveiled. Recent reports indicate that ROS production is a biphasic process and that PI3K γ plays a key role in the initiation of the first phase but not of the second phase. Nonetheless, the second phase of ROS production depends on the first one, which is mediated uniquely by PI3K γ activity (26). This suggests a crucial role of PI3K γ in ROS production but in a condition of GPCR-dependent activation that likely does not represent the main mechanism occurring *in vivo*. Indeed, in infection and inflammation, ROS production is triggered by opsonized particles that can drive a PI3K γ -independent respiratory burst. This implies that PI3K γ does not play a crucial role in infection-mediated production of ROS and that other PI3K isoforms might be more important in this context (10, 27).

On the other hand, the absence of PI3K γ causes a dramatic reduction in leukocyte chemotaxis towards GPCR agonists and in particular, chemokines. This was observed both *in vitro* and *in vivo* and led to the conclusion that PI3K γ has a prominent role downstream of chemokine receptors in mediating leukocyte migration and pathfinding. *In vitro*, it has been reported that, compared to wild type controls, PI3K γ -null macrophages and neutrophils display reduced migration in response to several chemoattractants (17, 24, 25). In particular, PI3K γ -null macrophages show impaired directionality and reduced speed in migration towards monocyte chemoattractant protein-1 (MCP-1) in a Dunn chamber assay, where cells are directly visualized as they migrate in a chemotactic gradient (19). Similarly, recruitment of PI3K γ -null neutrophils and macrophages towards sites of inflammation is impaired *in vivo*. For example, granulocytes lacking PI3K γ are not recruited to *E. coli* seeded in the peritoneal

cavity (17, 24) and to lungs subjected to a model of sepsis (28). Consistently, PI3K γ is necessary for bronchoalveolar recruitment of granulocytes in lungs instilled with either chemokines or LPS (4, 29). The ablation of PI3K γ also reduces the severity of acute pancreatitis by blocking neutrophil infiltration within the pancreatic tissue at an early stage of the disease (30). Furthermore, PI3K γ inhibition protects from leukocyte infiltration of synovia in a murine model of rheumatoid arthritis (31).

Reduced chemotaxis in the absence of PI3K γ results not only in impaired innate immunity but also in deficits in adaptive immunity. For example, PI3K γ controls motility of dendritic cells (DCs), a leukocyte population that, by presenting antigens, controls T- and B-lymphocyte activation as well as immunotolerance. Normally, DCs reside in an immature state in peripheral tissues where they possess a sentinel function (32). During inflammation these cells encounter the antigens and start a process of maturation that promotes dendritic cell migration to the draining lymph nodes (33). Both immature and TNF-treated mature dendritic cells, obtained from PI3K γ -null mice, displayed impaired ability to migrate *in vitro* in response to various chemokines (34). Moreover, *in vivo*, it was demonstrated that PI3K γ -null CD34-derived dendritic cells reach the draining lymph nodes in a reduced number in comparison to wild-type cells. Similar results were obtained for resident cutaneous DCs lacking PI3K γ (34). The impaired migration of antigen-loaded PI3K γ -null dendritic cells from the skin to lymph nodes was associated with a reduced contact hypersensitivity and delayed hypersensitivity reactions.

Experimental evidence indicates that PI3K γ not only controls lymphocyte function indirectly by driving DCs activity but also directly by modulating T-cell migration. In agreement, *in vitro* migration of both CD4 and CD8 positive PI3K γ -null T-cells towards chemokine like CCL19, CXCL12 and CCL21 is significantly decreased in comparison to wild-type T-cells (35). In addition, PI3K γ might play a role in the recruitment of CD4⁺ memory T-cells to the site of inflammation (36). Despite these results, PI3K γ does not appear to be essential for T-lymphocyte chemotaxis as it has been demonstrated that its role in T-cell migration is at least secondary to that played by the Rac GEF DOCK2 (37, 38). Nonetheless, after GPCR stimulation, PI3K γ is required for optimal T-cell chemotaxis: although the lack of PI3K γ has no effect on migration speed, it causes increased turning and reduced persistence in directional movement (38). Recent findings indeed suggest that, during T-cell migration, PI3K γ helps to maintain temporal directionality by regulating the organization of F-actin formation (38).

Altogether, these data indicate that, in inflammatory responses, the absence of PI3K γ function cannot be compensated by other class I PI3Ks and that PI3K γ plays a crucial non redundant role in leukocyte motility (4, 17, 25, 31, 39). Experimental evidence shows that PI3K γ controls leukocyte polarization and directional migration by regulating the spatial accumulation of PtdIns(3,4,5) P_3 . In this context, PI3K γ appears to be crucial for translating shallow gradients of chemotactic agents into differences of PtdIns(3,4,5) P_3 concentration and localization. Data obtained using confocal imaging and time lapse microscopy, demonstrate that PI3K γ -null neutrophils lose directionality during fMLP-induced chemotaxis because they fail to polarize and form

a normal migration front (18). Similar results were obtained for macrophages, thus suggesting a PI3K γ involvement in regulating an efficient response to gradient sensing (5, 19, 40). In seeming contrast, analysis of PI3K γ function in neutrophil migration using more stringent experimental conditions reveals that this enzyme is not required for directional movement per se but rather for gradient-independent cell movement (41). Nonetheless, in these same experimental settings, PI3K γ remains responsible for the regulation of crucial aspects of neutrophil polarization, such as integrin-based adhesion and accumulation of polymerized F-actin at the leading edge. This key role of PI3K γ in organization of the migration front, suggests that this enzyme may not be responsible for the initiation of leukocyte polarization, but that it plays an important role in stabilization and amplification of this process (41). Indeed, chemoattractant-induced leukocyte polarization depends on the formation of the F-actin meshwork at the front but also on contraction of actomyosin at the back of the cell. This requires a complex interplay between Rho GTPases and the actin cytoskeleton. PtdIns(3,4,5) P_3 accumulation at leading edge of leukocyte has a pivotal role in the localization of the active GTPases and their effectors (42). In neutrophil-like HL60 cells, it has been demonstrated that the inhibition of PtdIns(3,4,5) P_3 accumulation prevents the activation of Cdc42 and reduces Rac activity, thus causing the formation of unstable pseudopods (43). This is consistent with the established role of PtdIns(3,4,5) P_3 as a mediator of positive feedback pathways that augment Rac activation at the front of the cell and eventually regulate F-actin polymerization. Furthermore it has been shown that PtdIns(3,4,5) P_3 and Cdc42 exert a stabilizing effect by strengthening pseudopods and by promoting activation of Rho-dependent actomyosin contraction at the back of the cell. Indeed, the inhibition of PI3K γ or Cdc42 activity disrupts this stabilizing effect leading to the formation of multiple transient pseudopods (43). In agreement, mouse macrophages lacking PI3K γ possess a normal Rac2 activity but impaired Rac1 kinetics (40), suggesting that PtdIns(3,4,5) P_3 controls the duration rather than the triggering of Rac activity. This is probably achieved by a PI3K γ -dependent control not only of Rac triggering but also of Rac deactivation. Consistent with this view, mice carrying a knock-in allele encoding a constitutively active PI3K γ do not polarize PtdIns(3,4,5) P_3 , do not form a leading edge, but show shortened Rac activation (5). Indeed, this PI3K γ mutant mediates the modulation of PtdIns(3,4,5) P_3 -dependent GAP activity that can involve the PH domain-containing ArhGAP15 (5). This event defines a PI3K γ -dependent molecular mechanism that limits and tunes cell polarization and directional motility (Fig. 2). Therefore, the involvement of PI3K γ in the control of PtdIns(3,4,5) P_3 production, small GTPases activity modulation, and F-actin containing pseudopod generation defines this enzyme as a crucial element necessary for efficient leukocyte chemotaxis (5, 40, 42, 43).

PI3K γ role in vascular endothelium

PI3K γ is also expressed in vessels, both in the endothelium and in the smooth muscle cells (22, 44). This finding suggests a role for PI3K γ in coordinating leukocytes and vascular wall elements during the development of the inflammatory process. Inflammation and allergy can induce edema and plasma leakage that involve both endothelial cells and resident cells, such as mast cells.

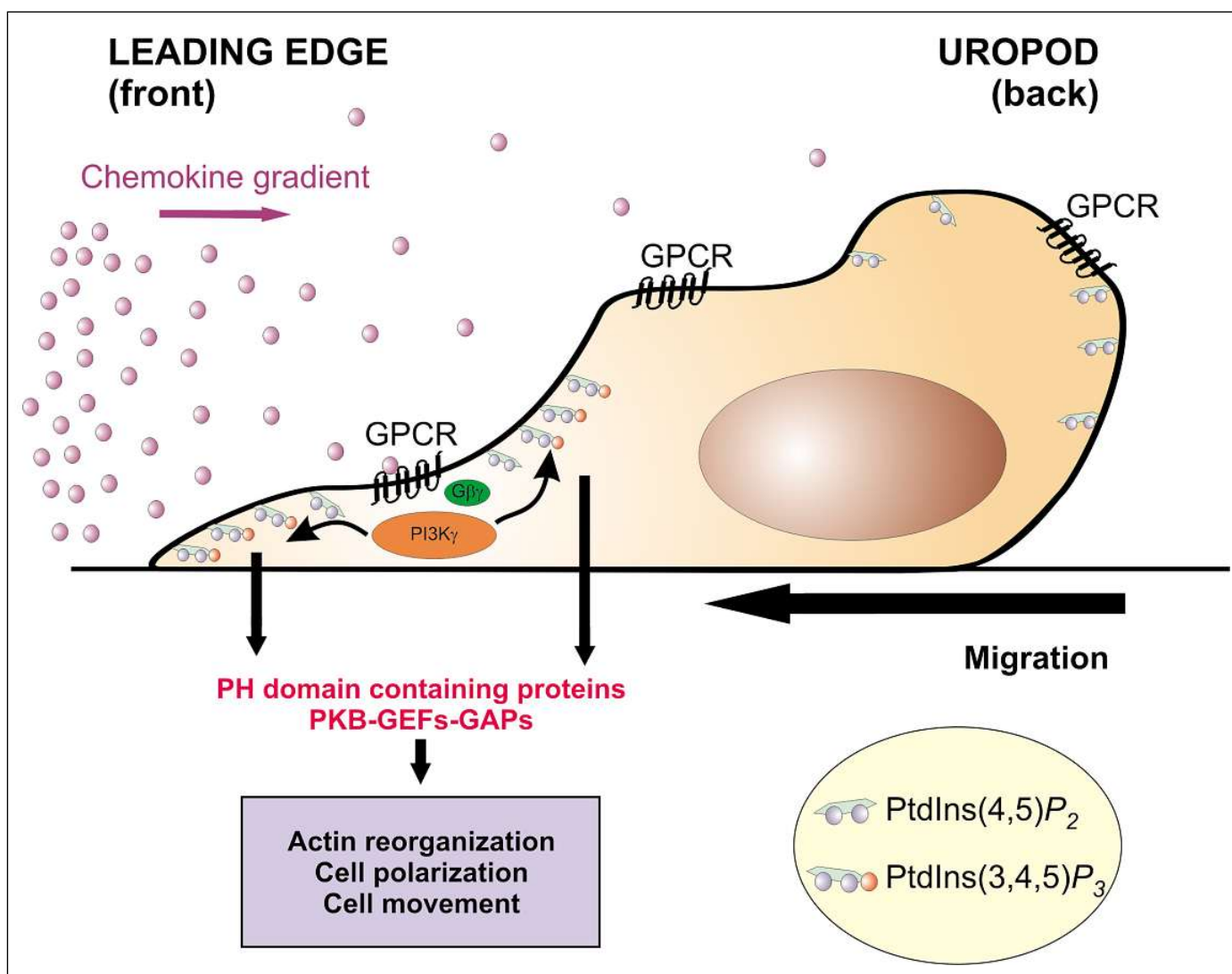


Figure 2: Simplified model of chemoattractant-induced leukocyte polarization and migration. By stimulating PtdIns(3,4,5)P₃ production, PI3K γ plays a crucial role in the stabilization of the leading edge of migrating leukocytes.

Mast cell activation induces systemic consequences like smooth muscle contraction and increased vascular permeability. The lack of PI3K γ protects animals from edema formation and increased vasopermeability induced by passive systemic anaphylaxis (45). Although anaphylaxis depends mainly on mast cell degranulation, it has been hypothesized that the protection exerted by the absence of PI3K γ is dependent not only on reduced mast cell reactivity but also on impaired endothelial and smooth muscle responses (46).

Puri et al. demonstrated the importance of the endothelial cell component of PI3K γ activity in promoting neutrophil interactions with the inflamed vessel wall (44). However, in endothelial cells, also PI3K δ is important for neutrophil adhesion and for subsequent transendothelial migration, thus suggesting that both PI3K δ and PI3K γ activity are required for efficient capture of neutrophils by cytokine stimulated endothelium (44, 47). In conclusion, in endothelial cells, PI3K γ and PI3K δ cooperate to regulate neutrophil extravasation, an event considered crucial for immune responses.

The PI3K γ role in vascular endothelium involves not only the control of leukocyte transmigration, but also the regulation of endothelial progenitor cells (EPC). EPCs are recruited from bone marrow to sites of ischemia and determine vasculogenesis. A recent study shows that both ischemia-induced angiogenesis as well as EPC mobilization and migration are impaired in the absence of PI3K γ (23). Experimental evidences indicate that PI3Ks regulate EPCs through phosphorylation of PKB/Akt and subsequent inactivation of FOXO (48–50). PKB/Akt and FOXO regulate eNOS activation (49), and in turn eNOS modulates EPC mobilization, migration and survival via generation of nitric oxide (NO) (51, 52). Madeddu et al. demonstrated that PI3K γ -null EPCs display dramatic defects in proliferation and survival as well as assimilation into endothelial networks and migration towards chemokines like SDF1. This phenotype of PI3K γ -null EPCs is primarily associated with reduced PKB/Akt activation, impaired eNOS phosphorylation and decreased NO production. Indeed, pre-treatment with a donor of NO like Glyco-SNAP is able to correct the migratory defect observed in PI3K γ -null

Table 1: PI3K γ inhibition and potential therapeutic implications.

Cell type	Targets	Clinical indications
Neutrophils	Migration (17) burst (26)	Septic peritonitis (25,31) Airway inflammation (29) Acute lung injury (ALI) (5) Adult respiratory distress (ARDS) (5) Obstructory pulmonary disease (COPD) (5) Joint inflammation (rheumatoid arthritis) (31)
Macrophages	Migration (17)	Septic peritonitis (25, 31) Obstructory pulmonary disease (COPD) (5) Joint inflammation (rheumatoid arthritis) (31) Systemic lupus erithematosus (36) Atherosclerosis (10)
T-lymphocytes	Migration (25) homing (35)	Glomerulonephritis (36) Systemic lupus erithematosus (36) Autoimmune diseases (35) Inflammatory diseases (35)
Dendritic cells	Migration (34)	Delayed type hypersensitivity (34) Allergic diseases (34, 45) Systemic lupus erithematosus (36)
Mast cells	Degranulation (45)	Passive systemic anaphilaxis (45) Asthma (45) Rheumatoid arthritis (31) Allergic diseases (34, 45)
Eosinophils	Migration (29, 45)	Allergy (45) Asthma (29, 45)
Smooth muscle	Vasoconstriction (22)	Inflammatory diseases (22) Hypertension (22)
Endothelial cells	Extravasation (44)	Anaphylaxis (10) Ischemia induced angiogenesis (23)

EPCs (23). These data thus imply an important role of PI3K γ in reparative angiogenesis. Interestingly, recent reports indicate that angiogenesis plays a detrimental role in diseases like arthritis and atherosclerosis (53, 54). The finding that PI3K γ function is involved in this process thus defines new implications for PI3K γ inhibition in the treatment of inflammatory diseases.

PI3K γ inhibition: therapy for inflammatory diseases

In mice, the absence of PI3K γ confers resistance to the development of several inflammatory pathologies including asthma, rheumatoid arthritis, allergy, systemic lupus erythematosus (SLE), airway inflammation, lung injury and pancreatitis (4, 13, 16, 31, 36, 46). In contrast, a recent paper reported that both genetic and pharmacological specific ablation of PI3K γ resulted in the impairment of lung inflammatory response after challenge with *S. pneumoniae* (55). This event causes a strong increment of the risk of lung bacterial infection. Nevertheless, despite the adverse effect that inhibition of PI3K γ might have on the perturbation of innate immune responses to infection, PI3K γ blockade still appears attractive for the treatment of pathologic inflammation. In fact, in the future, PI3K γ inhibition could represent a valuable alternative to currently available therapies for a large number of inflammatory diseases (Table 1).

Presently, several pharmaceutical companies are involved in the production of PI3K inhibitors with isoform selectivity. The first PI3K inhibitors described, wortmannin and LY294002, are not able to distinguish between different isoforms and thus, by acting on all class I PI3Ks, including those involved in insulin and growth factor receptor signaling, might lead to severe adverse effects. For this reason, selectivity appears a crucial requirement for PI3K inhibitors to be used in inflammatory diseases. Despite the fact that class I PI3Ks display overall similarity in the ATP-binding sites, design and production of isoform selective inhibitors has been demonstrated as a difficult but accessible goal. In particular, in the past three years, a large number of patents have been published that claim compounds able to selectively inhibit the PI3K γ isoform. Novartis described the first PI3K γ specific inhibitor, which consists of a derivative of 5-phenylthiazole (56). From this compound, Novartis has proposed other molecules derived from its optimization (57–59). The pharmaceutical group Pfizer developed thiazolidinedione derivatives (60–64) and Serono presented amino-bis-thiazoles and azolidinone derivatives (65–68). Accordingly to PI3K γ role in the control of inflammation, all these patents claimed their compounds as helpful in the treatment of a broad spectrum of autoimmune and inflammatory diseases.

Recently two different papers demonstrated the in-vivo efficacy of PI3K γ inhibitor AS605240 in the treatment of murine models of systemic lupus erythematosus (SLE) and rheumatoid arthritis, respectively (31, 36). SLE is a chronic inflammatory

disease which results in glomerulonephritis and renal failure. Conventional therapies for SLE consist of immunosuppressant and cytostatic agents that induce numerous side effects (69). Barber et al. showed that the intraperitoneal administration of the AS605240 compound in the MRL-lpr mouse model of SLE resulted in a reduced glomerulonephritis and in a prolonged lifespan of the animals. Moreover, after three months of treatment, AS605240 did not induce any adverse effect. Interestingly, mice recover even when treatment starts after they have already displayed symptoms of the disease (36). Similarly, rheumatoid arthritis is also a chronic systemic inflammatory disease that mainly affects joints. At present, efficacious therapies with limited side effects still represent an unmet need (70). Camps et al. demonstrated that oral treatment with AS605240 suppresses the progression of joint inflammation and reduces leukocytes infiltration in a mouse model of collagen-induced arthritis (31). The efficacy of AS605240 treatment supports PI3K γ inhibition as a promising approach that can effectively ameliorate chronic inflammatory disorders.

Experimental evidence shows that PI3K γ and PI3K δ cooperate in many immune cells. For this reason, both isoforms can be considered as valid targets for the pharmacological treatment of inflammatory diseases. Indeed, in some clinical situations, it can be useful to inhibit both isoforms simultaneously. For instance, since both PI3K γ and PI3K are involved in regulating mast cells, it might be promising to block both these isoforms in the treatment of degranulation and mast cell function diseases. Recently, the ICOS corporation suggested a method for the combined in-

hibition of PI3K γ and PI3K δ . This drug treatment prevents leukocyte accumulation into inflamed tissues by the inhibition of their transmigration across vascular endothelium (71). For the same reason, Targegen has described a new compound (TG100-115) able to inhibit together PI3K γ and PI3K δ . This compound was found to block edema formation and inflammation. Moreover, it reduced neutrophils recruitment in the ischemic tissue without affecting the positive angiogenic functions of endothelial cells (72).

These experimental data confirm PI3K γ as a promising target for the treatment of human inflammatory and autoimmune diseases. Though administration of such PI3K γ inhibitors might result in discouragement in situations, like infection, where fully functional immune responses are needed, the very large number of applications of PI3K γ blockade still support the search for such drugs. The beneficial effects of PI3K γ inhibition in conditions of pathological inflammatory responses appear so attractive that several researchers have compared the possible widespread use of a PI3K γ inhibitor to that of aspirin (16). The compounds described so far have not yet reached clinical use, but, with such high expectations, interesting developments will likely turn up in the near future.

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