Phosphoinositide 3-kinases and their role in inflammation: potential clinical targets in atherosclerosis?

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ABSTRACT

Inflammation has a central role in the pathogenesis of atherosclerosis at various stages of the disease. Therefore it appears of great interest to develop novel and innovative drugs targeting inflammatory proteins for the treatment of atherosclerosis. The PI3K (phosphoinositide 3-kinase) family, which catalyses the phosphorylation of the 3-OH position of phosphoinositides and generates phospholipids, controls a wide variety of intracellular signalling pathways. Recent studies provide evidence for a crucial role of this family not only in immune function, such as inflammatory cell recruitment, and expression and activation of inflammatory mediators, but also in antigen-dependent responses making it an interesting target to modulate inflammatory processes. The present review will focus on the regulation of inflammation within the vasculature during atherogenesis. We will concentrate on the different functions played by each isoform of PI3K in immune cells which could be involved in this pathology, raising the possibility that inhibition of one or more PI3K isoforms may represent an effective approach in the treatment of atherosclerosis.

PHYSIOPATHOLOGY OF ATHEROSCLEROSIS

Arteries present a common structural pattern consisting of three concentric tunica separated by elastic lamina layers. The innermost tunica intima delimits the vessel wall towards the blood flow and is composed of lining ECs (endothelial cells). Below, the tunica media is formed by a layer of circumferential vascular SMCs (smooth muscle cells) which maintain vasculature tone. Finally, the outer adventitia which consists mainly of connective tissue fibres blends the vessel into its surrounding [1].

Atherosclerosis is a multifaceted pathology affecting large-/medium-sized arteries and is an inevitable component of aging. Indeed, triggers of atherosclerosis, such as consuming a fatty diet, smoking, hypertension, dyslipidaemia, obesity, infections and stress, can initiate the pathological mechanisms that make the vascular endothelium dysfunctional. Disturbed ECs results in...
altered endothelium permeability, permitting lipoprotein accumulation within the intima and inducing EC molecule adhesion expression, which mediates leucocyte adhesion and infiltration. This intimal collection constitutes the ‘fatty streak’, which is asymptomatic and can evolve to advanced/complicated lesions depending on the inflammatory process. In the vessel wall, infiltrated monocytes mature into macrophages which accumulate modified lipoproteins through receptor endocytosis and are converted into foam cells. Simultaneously, T-cells direct their adaptive immune response. Cytokines and growth factors secreted by leucocytes, as well as ECs, promote proliferation and migration of SMCs, surrounding the lipid core with a protective fibrous cap and separating the prothrombotic lipid pool from luminal blood flow. In the intima, SMCs secrete extracellular matrix proteins, and this process contributes to the evolution of the lesion from a lipid-rich plaque to a fibroptic, and, in the long-term, calcified lesion [1,2].

During the 1990s, the balance between SMCs and inflammatory cells was emphasized as a strong determinant of plaque rupture and plaque stability and became a major notion in understanding the clinical manifestation of atheroma disease. In stable plaques, a thick cap consisting of SMCs and extracellular matrix covers the entire lipid core. These lesions are clinically silent and they rarely rupture. By contrast, the fibrous cap in unstable plaques is thin, especially at the shoulder lesion and contains few SMCs, but numerous inflammatory cells. These plaques are prone to rupture leading to thrombosis, followed by either occlusion or episodic plaque expansion. Moreover, during plaque development, remodelling of the artery takes place and consists of reactive changes in the underlying vessel wall to preserve a normal lumen and prevent flow occlusion. However, the degree of inflammation and cap thickness determine the risks of plaque rupturing [3]. Repeated ruptures increase stenoses and can promote myocardial infarction, stroke and gangrene in the peripheral vasculature.

Thus understanding of the atherosclerosis process shows that inflammation participates from atheroma inception, its development up until complications and places this vascular pathology as a chronic inflammatory disease.

INFLAMMATION IN ATHEROSCLEROSIS DEVELOPMENT

The inflammatory processes of atherosclerosis require both innate and adaptive immunity. Macrophages play a central role among immune cells involved in this pathology, since they take part in both the lipids core formation and the production of inflammatory mediators. In addition, accumulating evidence supports the important role of T-cells in the first steps of atherosclerosis, whereas B-cells are poorly represented in the intimal plaque and their role is limited to antigen presentation [4].

In addition, MCs (mast cells) and neutrophils have recently been identified in atherosclerotic lesions. MCs appear to play different functions in atherogenesis from endothelial alteration to final plaque destabilization [5], and neutrophils have been suggested to be involved in the pathology due to their ability to produce ROS (reactive oxygen species) via the NADPH oxidase pathway and, thus, promoting lipid peroxidation, endothelial dysfunction, inflammation, cell migration and apoptosis [6]. However, identification of neutrophils in atherosclerotic lesions, as well as their involvement in atherogenesis, have only recently been demonstrated [7,8].

With the development of mouse models of atherosclerosis, it has become possible to examine more carefully the role of immune responses in this disease. Among the most widely used mouse models of atherosclerosis are ApoE−/− [ApoE (apolipoprotein E)-deficient] and LDLr−/− [LDL (low-density lipoprotein) receptor-deficient] mice [9,10]. ApoE is a glycoprotein synthesized in the liver and macrophages, and is involved in lipid metabolism. Deletion of the ApoE gene in mice leads to severe hypercholesterolaemia and spontaneous development of atherosclerotic lesions that are well-characterized and resemble lesions in humans from fatty streak lesions to fibrous cap formation. LDLr−/− mice, a model of familial hypercholesterolaemia, have a more modest lipoprotein abnormality than the ApoE−/− mice and develop atherosclerotic lesions only after a high-fat/high-cholesterol diet. As with ApoE−/− mice, lesions developed by these mice appear morphologically identical with those found in humans.

Inflammation in early atherosclerotic lesions (fatty streaks)

Shortly after VCAM-1 (vascular cell-adhesion molecule-1) induction on ECs (Figure 1A), monocytes and T-cells pass through the arterial intima by diapedesis. Various chemokines contribute to this process, such as MCP-1 (monocyte chemotactic protein-1) and IL-8 (interleukin-8), both involved in macrophage recruitment, and IFN-γ (interferon-γ)-inducible chemokines, including IP-10 (inducible protein-10), Mig (monokine induced by IFN-γ) and I-TAC (IFN-inducible T-cell α-chemoattractant), which are involved in T-cell activation. As an example, mice susceptible to atherogenesis and lacking MCP-1 or its receptor CCR2 (CC chemokine receptor 2) are defective in atherogenesis, with a decrease in mononuclear phagocyte accumulation [11,12].

Production of M-CSF (macrophage colony-stimulating factor) by ECs and SMCs favours monocyte differentiation into pro-atherogenic macrophages, which accumulate lipids and secrete pro-inflammatory mediators within the intima. Lipid uptake by macrophages is mainly attributed to their ability to internalize oxidized or
Figure 1  Inflammatory cells involved in atherosclerosis

(A) Inflammation in fatty streaks. After VCAM-1 induction on ECs, leucocytes (monocytes, T-cells and MCs) enter the arterial intima by diapedesis. Differentiated macrophages accumulate modified lipoprotein (oxLDL) by scavenger receptors and are converted into foam cells. T-cells then direct their adaptive immune response. In parallel, MCs release granule components which enhance endothelial permeability to plasma lipoproteins and inhibit cholesterol efflux in macrophages. ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; VLA-4, very late antigen-4. (B) Inflammation in plaque stabilization. In innate immunity, macrophages express TLRs which bind to LPS, Hsp60, oxLDL etc. and induce pro-inflammatory cytokines, proteases, and pro-apoptotic and pro-coagulant mediators. Interaction between TCRs and antigens carried by MHC class II macrophages activate T-cells. After IL-12, IL-15, IL-18 and IFN-γ secretion, T-cells direct a pro-inflammatory Th1 response, whereas IL-4 secretion results in a Th2 anti-inflammatory response able to stimulate B-cells (BL), which could produce antibodies against oxLDL. In the same way, MCs secrete TNF-α, which induces EC detachment, and other granule components which reduce collagen production and influence SMC proliferation/apoptosis. Together, innate and adaptive immune responses could influence the atherosclerotic plaque towards a stable or unstable fate.
glycated lipoproteins through scavenger receptors such as CD36, CD68, CXCL16 (CXC-chemokine ligand 16), LOX-1 (lectin-type oxLDL (oxidized LDL) receptor-1) and SR-A (scavenger receptor-A), which are highly expressed on their cell surface. As these receptors are poorly regulated, macrophages accumulate cholesterol and become foam cells, which are highly present in early fatty streak lesions. According to these results, mice defective in the M-CSF, G-CSF (granulocyte colony-stimulating factor), SR-A or CD36 are all defective in atherogenesis [13]. In parallel with foam cell formation, oxLDL, LPS (lipopolysaccharide) and Hsp60 (heat-shock protein 60) interact with TLRs (Toll-like receptors), which are innate immune recognition receptors, leading to macrophage activation. These activated macrophages disrupt vascular cell functions by synthesizing and releasing pro-inflammatory cytokines, chemokines, growth factors [FGF (fibroblast growth factor) and PDGF (platelet-derived growth factor)], MMPs (matrix metalloproteinases), ROS, eicosanoids and tissue factor. All of these factors are involved in the progression of atherosclerosis and plaque destabilization (see below). The importance of macrophage function in atherosclerosis can be illustrated by the observation that hypercholesterolaemic Op/Op (Csf1−/−) mice lacking differentiated macrophages in tissues due to a mutation in the M-CSF gene (Csf1) develop only few atherosclerotic lesions [14].

Adaptive immunity then takes over, with T-cell activation by interaction with antigens presented by macrophages expressing MHC class II molecules (oxLDL, Hsp etc.). Lymphocytes within atherosclerotic lesions are mainly CD4+ lymphocytes and most of them are αβ T-cells with a small proportion of γδ T-cells. The importance of lymphocytes in atherosclerosis development has been clearly demonstrated in atherosclerotic mice. LDLr−/− mice crossbred with lymphocyte-deficient Rag1−/− (recombination activating gene 1) mice have a dramatic reduction in fatty streak lesions [15]. Likewise, immunodeficient ApoE−/−/scid-scid mice have reduced atherosclerotic lesions compared with ApoE−/− mice. Transfer of CD4+ T-cells in these immunodeficient mice not only reverses this protection, but also dramatically induces atherosclerosis [16]. Previous studies have demonstrated that the Th1 subset of CD4+ is the predominant type of lymphocytes found in atherosclerotic lesions, probably owing to local production of IL-12, IL-15 or IL-18 [17,18]. Th1 lymphocytes have a pro-atherogenic effect associated with their production of IFN-γ, as it has been shown that ApoE−/−/IFN-γ−/− mice have smaller lesions than those observed in ApoE−/− mice [19]. Production of IFN-γ and TNF-α (tumour necrosis factor-α) by T-cells induces macrophage activation, production of proteases and other pro-inflammatory mediators, EC activation, an increase in adhesion molecule expression, and inhibition of SMC proliferation and collagen production. Moreover, the anti-atherogenic effect of IL-10 [20–22], known to regulate Th1 and Th2 responses by limiting T-cell activation and differentiation in lymph nodes as well as suppressing pro-inflammatory responses in tissues, reinforces the importance of T-cells in the development of atherosclerosis [23]. The role of Th2-related responses in atherosclerosis is still controversial and there is no evidence to conclude that the Th2 response is invariably protective against atherosclerosis progression [18,24]. A novel functionally distinct subpopulation of T-cells, called regulatory T-cells, has been shown to exert important regulatory functions in atherosclerosis. Ait-Oufella et al. [25] showed that naturally arising CD4+CD25+ regulatory T-cells are powerful inhibitors of atherosclerosis in several mouse models. Thus, although T-cells are not abundant, they appear to strongly modulate lesion initiation and the early progression of atherosclerosis [15], and to contribute to the regulation of plaque stability.

It has been shown recently that MCs also participate in the development of atherosclerosis [5]. Indeed, MC-deficient mice (LDLr−/− × KitW−/−/Wsh/Wsh) had decreases in lesion size, lipid deposition, T-cell and macrophage numbers, cell proliferation and apoptosis, but increased collagen content. Kovanen et al. [26] have proposed many roles for MCs in atherogenesis, such as modification of endothelial permeability or an involvement in foam cell formation. The chemokines mainly responsible for recruitment of MC progenitors into the intima are SCF (stem cell factor) and eotaxin or CCL11, a CC chemokine overexpressed in atherosclerotic lesions [27]. Once in the intima, MCs undergo degranulation and release TNF-α, heparin, histamine and the serine proteases tryptase and chymase. Histamine from MC granules may locally induce enhanced endothelial permeability, leading to increased plasma lipoprotein infiltration. Moreover, heparin proteoglycans released from MC granules bind LDL particles which undergo proteolysis by chymase. These LDL particles fuse into larger lipid droplets that are phagocytosed by macrophages leading to foam cell formation [28]. In addition, MC chymase and tryptase can actively degrade HDL (high-density lipoprotein) and poorly lipidated HDL (preβ-HDL), thereby inhibiting cholesterol efflux from macrophage foam cells [26]. Therefore MCs may both stimulate cholesterol uptake and inhibit cholesterol efflux, leading to intimal foam cell and fatty streak lesion formation.

### Inflammation in advanced and complicated atherosclerotic lesions

Fatty streaks can progress into mature atherosclerotic plaques (Figure 1B) that contain more complex lesions characterized by a lipid-rich core covered by a fibrous cap and a large number of activated inflammatory cells, particularly T-cells and macrophages. Other cell types are present such as dendritic cells, MCs, a few B-cells and
natural killer T-cells. These immune cells are in an activated state and produce pro-inflammatory cytokines such as IFN-γ and TNF-α.

Macrophages play major roles in plaque destabilization through the production of various enzymes, activators, inhibitors and bioactive mediators. For example, macrophages express and release MMPs, which digest the plaque's matrix and thus impair its stability. This destabilization can also result from the induction of SMC apoptosis by macrophages requiring direct cell–cell contact or proximity and partly due to Fas/Fas-ligand interactions [29]. On the other hand, apoptosis of macrophages themselves induces necrotic core formation, reinforcing plaque fragility [30].

In addition to macrophages, T-cells localize at sites of plaque rupture. Th1 cytokines (IFN-γ and TNF-α) inhibit the proliferation of SMCs and the production of collagen by SMCs, thereby reducing stability of the plaque. Moreover, pro-inflammatory cytokines, such as IL-1 or TNF-α, can stimulate MMP activity and their production by SMCs [31,32]. IFN-γ inhibits SMC proliferation and can also, together with TNF-α and IL-1, promote apoptosis of SMCs [33], increasing the risk of plaque rupture.

MCs have also been identified in advanced atherosclerotic lesions and at the sites of plaque rupture or erosion [34]. The number of degranulated MCs is especially pronounced in the shoulder regions of athromata, the predilection sites for atheromatous rupture. Moreover, the number of MCs tends to increase with the clinical severity of coronary syndromes [26]. These findings argue for the possibility that MC degranulation contributes to the vulnerability of plaques [35]. MCs may also participate in plaque erosion by inducing apoptosis of ECs via the secretion pro-apoptotic factors, such as TNF-α, thereby inducing the detachment of ECs from the cap. Moreover, MCs can contribute to matrix degradation by secreting proteases (chymase and tryptase) that activate MMPs, promoting collagen degradation and plaque destabilization. For example, studies have shown that human chymase and tryptase activate pro-MMP-1 [36] and pro-MMP-3 [37] respectively. In addition, chymase could act directly on SMC function by inhibiting collagen synthesis [38] or by inducing their apoptosis [39]. These effects would weaken the cap and predispose the plaque to rupture.

Recent findings have shown an accumulation of neutrophils in atherosclerotic lesions [7,8]. Their role in atherogenesis was clearly demonstrated in neutrophil-depleted ApoE−/− mice which develop reduced atherosclerotic lesions [8]. Moreover, analysis of neutrophils in human atherosclerotic plaques showed a high number of NEP (neutral endopeptidase)-positive neutrophils in ruptured plaques compared with eroded plaques, suggesting a role for this cell type in plaque destabilization [40].

The most important consequences of atherosclerosis, such as myocardial infarction and stroke, are caused by thrombosis. Thrombus formation is driven by plaque rupture or erosion and, in human coronary atheroma, the numbers of macrophages, activated T-cells and MCs are increased at these sites of rupture or erosion [34,41]. This trio of pro-inflammatory cells work together, supporting a link between immunity and thrombosis in atherogenesis.

Thrombus formation starts with the coagulation cascade, which involves activated platelets and thrombin formation. In fact, inflammation processes stimulate tissue factor expression on cells within the plaque core (macrophages and SMCs), and rupture of the fibrous cap exposes the thrombogenic core to circulating platelets, leading to the aggregation process and subsequent artery occlusion. In addition to its well-recognized role as an effector of the coagulation cascade, thrombin is also linked to inflammatory pathways of atherosclerosis by directly activating vascular cells such as ECs, SMCs and macrophages [42]. Thus thrombotic processes could amplify inflammation, and inflammation promotes further thrombosis, creating an amplification loop that could maintain atherogenic signals.

**FAMILY OF PI3Ks (PHOSPHOINOSITIDE 3-KINASES)**

Inflammatory processes are relayed by a broad range of receptors at the cell surface, such as cytokine receptors, chemokine receptors and other growth factor receptors, but their activation could lead to common pathways of intracellular signalling. Among these shared intracellular events, activation of the family of PI3Ks has been described downstream of a large variety of immune receptors. Members of this family, producing 3-phosphoinositides and especially PtdIns(3,4,5)P_3, are key enzymes of intracellular signalling involved in many biological effects, such as proliferation, migration, metabolic homeostasis and cell survival, implicating PI3K in different pathologies including inflammatory and cardiovascular diseases. On the basis of their structural characteristics and substrate specificity, PI3K are divided into three classes referred to as class I, II and III (Figure 2) [43,44].

The class I PI3K family, the sole members of which are able to produce PtdIns(3,4,5)P_3, is divided into two subclasses. Class IA is characterized by a p110 (α, β or δ) catalytic subunit and a p85 regulatory subunit, whereas class IB is constituted by a single protein (p110γ or PI3Kγ) that could be linked to an adaptor protein (p101 or p84/p87) [45,46].

Each member of the class I PI3K family is activated by distinct pathways based on the cell type and the cell-surface receptor. The p85 subunit of class IA possesses the distinctiveness to bind consensus YXXXM motifs when they are phosphorylated on tyrosine residues. This motif is found on the intracytoplasmic tail of tyrosine
kinase receptors or adaptor molecules conferring to class IA PI3Ks an involvement downstream of tyrosine kinase receptor activation or tyrosine-kinase-associated receptor activation. Thus class IA PI3Ks are activated downstream of tyrosine kinase receptor stimulation, such as EGFR (epidermal growth factor receptor) [47], PDGFR (PDGF receptor) [48], FGFR (FGF receptor) [49], GHR (growth hormone receptor) [50,51], IGFR (insulin-like growth factor receptor) [52], insulin receptor [53] and different IL receptors [54]. In immune cells, this class is recruited after TCR (T-cell receptor), BCR (B-cell receptor), natural killer cell stimulatory receptors, Fc receptors and TLR activation [55]. Cross-linking of the immunoreceptor leads to activation of tyrosine kinases that are responsible for the phosphorylation of ITAMs (immunoreceptor tyrosine-based activation motifs). SH2 (Src homology 2)-containing tyrosine kinases recruited to this motif then create PI3K-docking sites on co-stimulatory receptors, such as CD28 on T-cells and CD19 on B-cells, or on adaptor molecules interacting with LAT (linker for activation of T-cells) [56].

Nevertheless, there is an exception concerning PI3Kβ activity. Although the structure of this isoform predicts it to be activated downstream of tyrosine kinase receptors, recent results from mouse mutants expressing a catalytically inactive PI3Kβ showed a more effective activation of PI3Kβ by GPCRs (G-protein-coupled receptors) than by tyrosine kinases [57–59]. The insulin receptor appears to be the sole tyrosine kinase receptor to involve PI3Kβ in maintaining long-term signalling [58].

PI3Kγ, the sole member of class IB, is activated by the direct binding of the catalytic and regulatory subunits of PI3Kγ to the βγ subunits of heterotrimeric G-proteins that could be potentiated by Ras-GTP. This distinctiveness confers to the protein an involvement downstream of GPCRs, such as chemokine receptors.
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Figure 3 Major PI3K functions in immune cells involved in atherosclerosis

The PI3Kγ isoform plays multiple functions in all haemopoietic cells involved in atherosclerosis, especially in the recruitment of immune cells, but it also has specific functions, such as in MC degranulation, platelet aggregation, the neutrophil oxidative burst and T-cell functions. The major effects of PI3Kδ are found in MC and T-cell functions and, to a lesser extent, in the neutrophil oxidative burst. The PI3Kδ isoform plays a major role in platelet aggregation, but could also be involved in the negative regulation of inflammatory processes. Although the PI3Kε isoform is expressed ubiquitously, no immune function has been described for this isoform.

Class II PI3Ks contain a C2 domain, which gives their name (PI3K-C2) to the members of this class of PI3K, and a PX domain (Phox homology domain). This domain is able to bind the two lipid products of PI3K-C2 PtdIns(3)P and PtdIns(3,4)P2 to localize these PI3K to the plasma membrane [60]. Class III PI3Ks contain a single member that is the homologue of Saccharomyces cerevisiae Vps34 that generates only PtdIns(3)P [61].

The physiological function of individual isoforms of PI3K have been demonstrated by the generation of genetically modified mice. The ubiquitous pattern of expression of PI3Kα or PI3Kβ leads to embryonic lethality of mice lacking catalytic subunits p110α [62] or p110β [63], demonstrating that both isoforms are necessary for development. By contrast, mice lacking p110γ or p110δ are viable and fertile with no apparent specific phenotype [64–67]. Indeed, these two isoforms are essentially expressed in the haemopoietic system and are restricted to more specialized functions. Thus PI3Kγ and PI3Kδ, positively involved in the control of inflammatory responses, represent interesting targets in pharmacology.

FUNCTIONS OF PI3K IN INFLAMMATION (Figure 3)

PI3K and inflammatory cell recruitment

Leucocytes accumulate at the site of inflammation by directional migration called chemotaxis generated by different types of molecules. Experiments of microinjection of specific antibodies directed against PI3K have demonstrated the involvement of the β and δ isoforms in migration induced by CSF [68]. In vitro experiments have also shown a role for PI3Kδ in controlling the directional movement of neutrophils [69]. Nevertheless, in vivo experiments appear to indicate that PI3Kγ is preferentially involved in leucocyte migration. In PI3Kγ-deficient mice, recruitment of neutrophils and macrophages is reduced in a mouse model of peritonitis induced by bacterial injection [64]. Furthermore, a selective inhibitor directed against PI3Kγ decreased neutrophil recruitment in a mouse model of peritoneal chemotaxis towards RANTES (regulated upon activation, normal T-cell expressed and secreted) [70].

PI3Kγ has also been shown at a lower level in ECs, where it plays an important role in supporting neutrophil interaction with the inflamed vessel wall. Using PI3Kγ−/− (PI3Kγ-deficient) mice reconstituted with wt (wild-type) neutrophils, Puri et al. [71] demonstrated a 45% reduction in the accumulation of neutrophils in an acute lung injury model. This appears to be due to a perturbation of selectin-mediated adhesion since, in PI3Kγ−/− microvessels, wt neutrophil attachment was reduced by approx. 70% in response to TNF-α. This modification in adhesion was increased by a deficiency in p110δ, suggesting that both PI3K class IA and PI3K class IB are required for efficient neutrophil recruitment by endothelium in response to cytokine stimulation [71].
PI3K activity is also involved in T-cell migration, but the isoform involved is still controversial. Indeed, in vitro studies have demonstrated that the migration of PI3Kγ-deficient CD4+ and CD8+ T-cells to CCL19 and CXCL12, and to low doses of CCL21, was reduced by 30–65%, whereas p110δ activity appeared to be dispensable [72,73].

In contrast, recent observations using multi-photon intravital microscopy revealed that a lack of PI3Kγ had negligible effects on migration velocities, but resulted in increased turning angles of T-cells [74]. Moreover, systemic lupus induced by an enhanced activation of PI3K class IA in T-cells was ameliorated by PI3Kγ-deficiency without any effects on T-cell invasion, suggesting that PI3K class IA, but not PI3K class IB, was involved in T-cell migration in vivo [75]. More recently, two groups have shown a role of p110δ in T-cell trafficking [76,77]. Indeed, it has been proposed that PI3Kδ activation mediates antigen-receptor-induced and cytokine-induced down-regulation of CD62L (L-selectin). This adhesion molecule regulates lymphocyte re-circulation. A kinase-inactive mutant of p110δ and PI3K inhibitors prevented both the proteolytic cleavage pathway and transcriptional mechanisms that control CD62L expression in activated T-cells [76]. Moreover, Jarmin et al. [77] have shown that T-cells from mice expressing catalytically inactive p110δ lost susceptibility to TCR-induced migration and failed to localize efficiently to antigenic tissues. These results indicate that p110δ is involved in T-cell trafficking and that pharmacological inhibition of this kinase could decrease T-cell-mediated inflammation.

**PI3K in T-cell function**

In addition to its role in T-cell migration, PI3K has also been involved in lymphocyte function. Lymphocytes respond to antigen by a direct binding through BCRs and TCRs or by interaction with the Fc region of immunoglobulins through the Fc receptor family. Class I PI3Ks could be activated by all of these receptors. In T-cells, activation of PI3K occurs after TCR cross-linking. This activity is enhanced by CD28 or IL-2 co-stimulation. However, the isoform involved in this process is controversial. Alcázar et al. [78] have demonstrated that, after TCR ligation, PI3Kγ interacts with Gαq/11, tyrosine kinases and ζ-associated proteins to produce 3-phosphorylated polyphosphoinositides at the immunological synapse. On the contrary, using an imaging approach to visualize PI3K signalling in single T-cells, Garçont et al. [79] have shown that PI3Kγ is not required for antigen-dependent PI3K activity and did not observe any defects in proliferation or cytokine secretion in p110γ−/− CD4+ T-cells, as demonstrated by Sasaki et al. [65]. They showed that PI3K activity at the T-cell–antigen-presenting cell contact area was dependent on the p110δ isoform. CD28 enhanced PtdIns(3,4,5)P3 production at this site independently of its YMNM motif required to interact with the p85/p110 heterodimer, suggesting that the heterodimer interacts with CD28 by another way. Nonetheless, PI3Kδ and PI3Kγ are involved together in thymocyte selection, where the combined activities of the two catalytic subunits were required for normal T-cell development [65,80].

**PI3K and activation of microbialicidal activity**

After recruitment to the inflamed site, neutrophils and macrophages generate ROS by activation of NADPH oxidase [81]. These ROS are involved not only in the killing process combating bacterial and fungal infections directly through the damaging actions of oxygen radicals, but also through the activation of phagosomal proteases. The PI3Kδ isoform plays an important role in this process, since the production of ROS induced by fMLP (N-formylmethionyl-leucyl-phenylalanine) in primed neutrophils was dramatically reduced in the absence of PI3Kγ [64–66]. The use of pharmacological inhibitors directed against different isoforms of PI3K has permitted the demonstration that ROS production is under the dependence of the sequential activation of different isoforms of PI3K. Stimulation of TNF-α–primed human neutrophils with fMLP results in the biphasic activation of PI3K. The first phase is strictly dependent on PI3Kγ, whereas the second one, which is entirely dependent on the first, requires activation of PI3Kδ and partially PI3Kα and PI3Kβ [82].

**Regulation of pro-inflammatory cytokine secretion by PI3K**

Even if most of the events recruiting PI3K activity in immune cells are involved in pro-inflammatory processes, in certain cases PI3K activity could regulate cytokine secretion to act as a limiting factor in inflammation. Upon bacterial infection, antigen-presenting cells, such as macrophages and dendritic cells, recognize PAMPs (pathogen-associated molecular patterns) using cell-surface TLRs. The PI3K pathway has been shown to be activated by various TLR ligands and can function as a positive or negative regulator of TLR responses depending on the cell type and the TLR used [83]. In sepsis, one of the most potent inflammatory agents LPS, a component of Gram-negative bacteria membranes, induces the production of inflammatory mediators and cytokines, such as IL-1α, IL-1β, IL-6 and TNF-α, via the activation of TLR-4 and the transcription factors Egr-1, AP-1 (activator protein-1) and NF-κB (nuclear factor κB) [84]. TLR-4 triggering induces the concomitant activation of PI3K, which is able to negatively regulate the production of inflammatory mediators in dendritic cells and human monocytes [83,85,86]. This negative regulation of inflammatory processes by PI3K has also been observed in mouse resident peritoneal macrophages stimulated...
with LPS. In this model, chronic administration of E2 (17β-oestradiol) to ovariectomized mice increases the expression of IL-1β, IL-6 and IL-12p40 in resident peritoneal macrophages in response to LPS ex vivo by altering the TLR-4 pathway. E2 treatment results in the inhibition of PI3K activity in LPS-activated macrophages, whereas NF-κB p65 transcriptional activity was concomitantly increased [87]. These results suggest that PI3K could modulate TLR signalling, having a role in the gate-keeping system to prevent excessive innate immune response. This function is probably mediated by a class IA PI3K as introduction of a vector producing short hairpin RNA targeting the p110β catalytic subunit in the mouse macrophage cell line Raw264.7 also enhanced the TLR-mediated response [88].

**Activation of MCs and eosinophils by PI3K**

MCs and eosinophils are, in addition to neutrophils and macrophages, essential in fighting against infections and parasites and could participate in the development of inflammatory pathologies, such as atherosclerosis, by releasing histamine-containing granules and immune mediators. MCs are activated by cross-linking of the IgE-receptor-induced allergen–IgE complex. This event leads to the activation of tyrosine kinases that phosphorylate ITAMs as well as Gab2 (Grb2 (growth-factor-receptor-bound protein 2)-associated binder 2) [89], creating docking sites for class I PI3Ks. PtdIns(3,4,5)P3 production in this context is necessary for Btk (Bruton tyrosine kinase) and PLCγ (phospholipase Cγ) activation, causing the opening of Ca2+ channels and granule release [90,91]. It is now proposed that p110δ and p110γ could be recruited in this pathway. p110δ could be recruited by the docking site mentioned above and could be responsible for a first wave of PtdIns(3,4,5)P3 production [92], and p110γ, recruited by an autocrine mechanism involving the sequestration of GPCR agonists, such as adenosine, could be necessary to obtain a full response in MC degranulation [94]. Consistent with these findings, inhibition of PI3Kδ [92,94] or PI3Kγ [93,95] in vivo induces a decrease in the anaphylactic allergic response in mice; however, recent studies using PI3Kγ- and PI3Kδ-specific inhibitors, as well as PI3Kγ- or PI3Kδ-deficient mice, have shown that PI3Kδ, but not PI3Kγ, was involved in the allergen-induced allergic response in vivo [96]. One explanation for the discrepancies between the studies could be the differences in the sensitization protocol. Although Laffargue et al. [93] used intravenous IgE and antigen injection, Ali et al. [92] performed local intradermal administration of IgE and antigen. We suggest that the dose of antigen in tissue differs between the two protocols and that the high dose used in the intradermal protocol masks the potentiation of the response induced by GPCR agonists.

Moreover, PI3Kγ activity appears to be responsible for the maintenance of eosinophilic inflammation in vivo [97], reinforcing the central role of this isoform in allergic pathology.

**Control of platelet function by PI3K**

Platelets play critical roles in thrombosis by adhering to and aggregating at sites of vascular injury. These two mechanisms are controlled by intracellular pathways, with growing evidence suggesting a major role for PI3K in these processes. Although all PI3K isoforms are expressed in platelets, only the PI3Kβ and PI3Kγ appear to play important roles in platelet activation. Indeed, p85-deficient mice, which have a large decrease in p110α, p110β and p110δ expression levels [98-100], have no defects in platelet aggregation in response to soluble agonists, such as ADP, TXA2 (thromboxane A2), thrombin or vWF (von Willebrand factor), suggesting a compensation by PI3Kγ [98]. Nevertheless, platelet aggregation was modified in response to collagen, suggesting a role of class IA PI3Ks in GPVI (glycoprotein VI) signalling [98]. Consistent with these findings, p110δ-deficient platelets had only a slight decrease in platelet aggregation in response to a low concentration of CRP (C-reactive protein), without any modification in response to the other classical stimuli [101].

The absence of a model with a specific deletion of p110α or p110β in a megakaryocyte lineage does not allow the role played by these two isoforms in platelet function to be dissected. To investigate the role of PI3Kβ, Jackson et al. [102] used a specific p110β inhibitor and demonstrated that this activity was necessary to sustain integrin αIIbβ3 activation and aggregation upon ADP stimulation and in response to low levels of other soluble agonists. Moreover, the PI3Kβ inhibitor prevented the formation of stable integrin adhesion contacts, leading to defective platelet thrombus formation. Interestingly, PI3Kγ-deficient mice have a defect in platelet aggregation in response to ADP and to threshold concentrations of collagen [103,104]. Studies of the molecular mechanisms showed a decrease in PKB (protein kinase B)/Akt phosphorylation and αIIbβ3 fibrinogen receptor activation in the absence of PI3Kγ downstream of P2Y12 receptor activation. These events did not modify bleeding time, but protected mice against thromboembolism induced by ADP. These studies suggest a role for multiple PI3Ks in this pathway and place PI3Kβ and PI3Kγ as potential pharmacological targets in antithrombotic therapy.

**TARGETING A PI3K ISOFORM IN ATHEROSCLEROSIS?**

Many drugs used in cardiovascular therapy have anti-inflammatory properties in addition to their targeting actions on modifying risk factors, such as hypertension and hyperlipidaemia [105]. Among them, ACEIs (angiotensin-converting enzyme inhibitors), ARBs (angiotensin
II type 1 receptor blockers), calcium channel blockers, the antiplatelet agent clopidogrel, and PPAR (peroxisome proliferator-activated receptor)-α and PPAR-γ activators appear to be involved in inflammatory pathways, but the clinical benefits of these effects remain unclear in cardiovascular diseases. In the same manner, atheroprotective action of statins, mainly used for their cholesterol-lowering effect, could also be attributed to their anti-inflammatory properties [106–109].

At the present time, great efforts are engaged in identifying novel and innovative drugs targeting inflammatory proteins for the treatment of atherosclerosis. Potential inflammatory targets are factors involved in the recruitment of leucocytes, including P-selectin, E-selectin, L-selectin, adhesion molecules, NF-κB, leucocyte integrins, cytokines (IL-1, IL-2, IL-6 and IL-18), and chemokines (MCP-1, RANTES, MIF (macrophage migration inhibitory factor), SDF-1 (stromal-derived factor-1)) and their receptors. Coagulation, fibrinolytic and complement activation cascades constitute other pro-inflammatory mediators that could be targeted for treating atherosclerosis. Other potentially interesting targets include the CB2 (cannabinoid 2) receptor that is expressed on macrophages and T-cells within atherosclerotic lesions. Treatment with a CB2 receptor agonist blocks atherosclerosis progression, probably by inhibiting the Th1 response and macrophage chemotaxis [110]. Interestingly, most of the PI3K isoforms are involved in these inflammatory processes.

Several pharmaceutical companies have developed PI3K inhibitors. Since the early 1990s, two pan-PI3K inhibitors, wortmannin and LY294002, have been largely used in many studies to analyse PI3K function. Wortmannin is a fungal metabolite initially isolated from Penicillium wortmannii [111] which inhibits all PI3Ks at nanomolar concentrations. By contrast, LY294002 is a synthetic compound [112] active at micromolar levels. These two compounds do not allow the discrimination between different PI3K classes, but have been useful tools to aid research and development of a new generation of compounds. Thus a panel of isoform-specific PI3K inhibitors has started to appear, and their structures have been recently reviewed by Marone et al. [113]. A series of compounds (AS-252424, AS-605240 and AS-604850) produced by Merck-Serono have been described that selectively inhibit PI3Kγ [114–117].

Initial studies have proposed that PI3Kγ-selective inhibitors possess immunomodulatory potentials, suggesting that PI3Kγ is a good candidate for drug development in chronic inflammation and autoimmune diseases [116]. In mouse models of rheumatoid arthritis, oral administration of selective PI3Kγ inhibitors successfully prevented the progression of joint inflammation and neutrophil accumulation [114]. Moreover, it has been demonstrated that intraperitoneal administration of a PI3Kγ inhibitor in a mouse model of systemic lupus erythematosus led to a reduction in pathogenic CD4+ memory cells, resulting in a blockade of glomerulonephritis and extended lifespan [115].

Recently, the role of PI3Kγ in atherosclerosis development has been demonstrated in vivo in mice by using different approaches [118,119]. Intraperitoneal administration of a specific PI3Kγ inhibitor (AS-605240; 10 mg/kg of body weight daily) significantly decreased early atherosclerotic lesions in ApoE−/− mice, as well as more advanced plaques in LDLr−/− mice fed a hypercholesterolaemic diet [119]. These results are in accordance with those reported by Chang et al. [118] showing that PI3Kγ−/−/ApoE−/− double-knockout mice have decreased lesion size compared with PI3Kγ+/+/ApoE−/−. PI3Kγ expression and activity are elevated in both human and mouse atherosclerotic lesions, especially in macrophage- and T-cell-rich regions. Moreover, deletion of the p110γ gene abrogates all detectable PKB/Akt activation as well as the PI3K-dependent phosphorylation of the downstream effector molecules FKHR [forkhead homologue in rhabdosarcoma; a member of the FOXO (forkhead box O) family of transcription factors], AFX (another member of the FOXO family of transcription factors), GSK3 (glycogen synthase kinase 3), p70 S6 kinase, S6 ribosomal protein and PKKθ (protein kinase Cθ) in atherosclerotic plaque macrophage/foam cells from ApoE−/− mice [118]. The involvement of PI3Kγ in inflammatory processes of atherogenesis has been demonstrated in chimaeric mice lacking PI3Kγ exclusively in immune cells with a decrease in the size of lesions (approx. 50%) [119]. Analysis of plaque cellular composition showed that the absence of immune cell PI3Kγ reduces macrophage (30%) and T-cell (50%) infiltration within atherosclerotic lesions as well as an increase in SMC and collagen content, suggesting a beneficial effect on plaque stability [119]. Taken together, these results clearly demonstrate that PI3Kγ has the potential to modulate inflammatory processes in the vascular wall responsible for multiple stages of atherosclerosis, such as fatty streak formation and plaque stability. The relative contribution of PI3Kγ from different immune cells could be interesting to dissect. The importance of PI3Kγ in T-cells and in eosinophils/MCs and the essential role of these two cell types in the initiation and progression of the pathology has led us to speculate that PI3Kγ from these two cell types is essential in atherosclerosis.

In addition to its immune functions, PI3Kγ has important functions in the cardiovascular system. Previous studies have demonstrated low levels of PI3Kγ in SMCs and in cardiomyocytes, where it controls vascular smooth muscle tone [120] and cardiac cell contractility [121,122]. Moreover, TargeGen has designed a new interesting compound, named TG100-115, able to inhibit both PI3Kγ and PI3Kδ. Studies using this inhibitor have demonstrated that PI3Kγ and PI3Kδ regulated infarct size after ischaemia/reperfusion injury [123]. TG100-115 has
entered clinical trials (phases I and II) for acute myocardial infarction [124]. Other PI3K inhibitors targeting all class I PI3K isoforms are entering phase I/II trials in cancer treatment (reviewed in [113]), but their potential side effects, especially on metabolism, could be a trade-off for their efficacy in the treatment of cardiovascular diseases.

CONCLUSIONS

Current therapies used in cardiovascular diseases target inflammation as a secondary part of their action. Advances in our understanding of the involvement of PI3K, in particular PI3Kγ, in inflammation have led to much interest in the development of specific inhibitors of each class I PI3K isoform. The next decade will see trials of agents that specifically target inflammation in the treatment of atherosclerosis. Inhibitors of specific class I PI3K isoforms could be one of these potential anti-inflammatory drugs useful not only in the treatment of atherosclerosis, but also in other chronic inflammatory diseases.

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Phosphoinositide 3-kinases and their role in inflammation


